EXHIBIT A

PENGAD-Bayonne, N.J.

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO: VRI BioMedical Ltd
Level 11 The BGC Centre
28 The Esplanade
PERTH WA 6000

RECEIPT IN THE CASE OF AN ORIGINAL DEPOSIT
issued pursuant to Rule 7.1 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified at the bottom of this page

Attention: Patricia Lynne Conway

NAME AND ADDRESS OF DEPOSITOR

I IDENTIFICATION OF THE MICROORGANISM	
Identification reference given by the DEPOSITOR:	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY:
Lactobacillus fermentum VRI 003	NM02/31074

II SCIENTIFIC DESCRIPTION AND/OR PROPOSED TAXONOMIC DESIGNATION

The microorganism identified under I above was accompanied by:

☒ a scientific description

☒ a proposed taxonomic designation

(Mark with a cross where applicable)

III RECEIPT AND ACCEPTANCE

This International Depository Authority accepts the microorganism identified under I above, which was received by it on 27th August 2002 (date of the original deposit) [1]

IV RECEIPT OF REQUEST FOR CONVERSION

The microorganism identified under I above was received by this International Depository Authority on (date of original deposit) and a request to convert the original deposit to a deposit under the Budapest Treaty was received by it on (date of receipt of request for conversion)

V INTERNATIONAL DEPOSITARY AUTHORITY

Name: AUSTRALIAN GOVERNMENT ANALYTICAL LABORATORIES Address: PO BOX 385 PYMBLE NSW 2073 AUSTRALIA Phone: (02) 9449 0111 Facsimile: (02) 9449 1653	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorised official(s) Date: 27th August 2002

1 Where Rule 6.4(d) applies, such date is the date on which the status of International Depositary Authority was acquired.

Form BP/4 (sole page)

BUDAPEST TREATY ON THE INTERNATIONAL
RECOGNITION OF THE DEPOSIT OF MICROORGANISMS
FOR THE PURPOSES OF PATENT PROCEDURE

INTERNATIONAL FORM

TO: Probiomics Ltd.
(formerly VRI BioMedical Ltd.)
Suite G09, 1 Central Avenue
Australian Technology Park
Eveleigh NSW 1430
Attention: Trisha Munoz

VIABILITY STATEMENT
issued pursuant to Rule 10.2 by the
INTERNATIONAL DEPOSITARY AUTHORITY
identified on the following page

NAME AND ADDRESS OF THE PARTY
TO WHOM THE VIABILITY STATEMENT
IS ISSUED

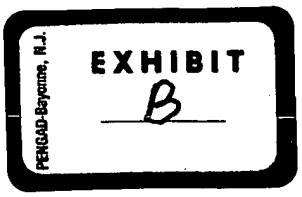
I DEPOSITOR	II IDENTIFICATION OF THE MICROORGANISM
Name: Trisha Munoz Address: Probiomics Ltd. (formerly VRI BioMedical Ltd.) Suite G09, 1 Central Avenue Australian Technology Park Eveleigh NSW 1430	Accession number given by the INTERNATIONAL DEPOSITARY AUTHORITY: NM02/31074 Date of the deposit or of the transfer: [1] 27th August 2002
III VIABILITY STATEMENT	
The viability of the microorganism identified under II above was tested on 3rd August 2005 [2]. On that date, the said microorganism was ☒ [3] viable ☐ [3] no longer viable	

1. Indicate the date of the original deposit or, where a new deposit or a transfer has been made, the most recent relevant date (date of the new deposit or date of the transfer).
2. In the cases referred to in Rule 10.2(a) (ii) and (iii) refer to the most recent viability test.
3. Mark with a cross the applicable box.

IV. CONDITIONS UNDER WHICH THE VIABILITY TEST HAS BEEN PERFORMED [4]
The culture was resuscitated from freeze-dried using Nutrient Broth, and grown anaerobically on MRS agar at 37°C / 24 hours.
V. INTERNATIONAL DEPOSITORY AUTHORITY

Name: NATIONAL MEASUREMENT INSTITUTE (FORMERLY AGAL) Address: PO BOX 385 PYMBLE NSW 2073 AUSTRALIA Phone: (02) 9449 0111 Facsimile: (02) 9449 1653	Signature(s) of person(s) having the power to represent the International Depositary Authority or of authorized official(s) ICR Emsley Date: August 4, 2005

4 Fill in if the information has been requested and if the results of the test were negative



Re: Lactobacillus fermentum – US Patent application

(viii) Claim 6: "resistant starch"

http://www.google.com.au/search?sourceid=navclient&ie=UTF-8&rls=GFRC,GFRC:2007-01,GFRC:en&q=resistant+starch

http://www.blackwell-synergy.com/doi/abs/10.1111/j.1541-4337.2006.tb00076.x?prevSearch=

http://www.nutrition.org.uk/upload/Resistant%20Starch(1).pdf

Google

resistant starch Search Advanced Search Preferences

Search: ◉ the web ○ pages from Australia

Web

Results **1 - 10** of about **202,000** for **resistant starch**. (**0.19** seconds)

Exhibit B

Resistant starch - 13 Dec
This **starch** is called **resistant starch** (RS) and many nutritionists think that it should be classified as a component of dietary fibre. ...
www.healthyeatingclub.com/info/articles/nutrients/resis**starch**.htm - 56k -
Cached - Similar pages - Note this

Health Report - 9/15/1997: **Resistant Starches**
Eating the recyclable package around your food as well as the food itself especially if it contains what are called probiotics Thats one of the dreams of Dr ...
www.abc.net.au/rn/talks/8.30/helthrpt/stories/s265.htm - 30k -
Cached - Similar pages - Note this

> ABC Radio National - Health Report Transcript
> **Resistant starches** are found in foods like rice, pasta, and potatoes. An Australian researcher who's been working in this field is Dr Jane Muir, ...
> www.abc.net.au/rn/talks/8.30/helthrpt/hstories/hr140803.htm - 11k -
> Cached - Similar pages - Note this

Hi-maize * Frequenty asked questions * What is **Resistant starch**?
Any **starch** which resists digestion and absorption in the small intestine and passes through to the large intestine, where it acts just like dietary fibre to ...
www.hi-maize.com/data.asp?id=205&lang_id=EN&country_id=US&category_id=1 - 10k -
Cached - Similar pages - Note this

Resistance starch in the diet
In the 1980s a new type of naturally occurring dietary fibre was discovered and named **resistant starch**, as it "resists" digestion in the small intestine and ...
aww.ninemsn.com.au/article.aspx?id=50824 - 63k - Cached - Similar pages - Note this

Resistant Starch - What is **Resistant Starch** - Sources - Benefits ...
What is **resistant starch**? Where can I get it? What Are the benefits of **resistant starch**? Questions about **resistant starch** answered.
lowcarbdiets.about.com/od/nutrition/a/**resistantstarch**.htm - 29k -
Cached - Similar pages - Note this

Resistant starch puts fat on top of hit list
THE idea of burning more fat and boosting your metabolism without dramatically changing your diet sounds too good to be true, but the latest research shows ...
www.ferret.com.au/articles/z1/view.asp?id=99882 - 31k - Cached - Similar pages - Note this

Resistant Starch - A Review - 13 Dec
ABSTRACT: The concept of **resistant starch** (RS) has evoked new interest in the .... **Resistant starch**. The term "**resistant starch**" was first coined by ...
www.blackwell-synergy.com/doi/abs/10.1111/j.1541-4337.2006.tb00076.x?prevSearch= -
Similar pages - Note this

Welcome to **Resistant Starch** » National **Starch** Food Innovation
New research published in the September issue of OBESITY included Hi-maize® **resistant starch** as the low glycemic carbohydrate More. ...
www.**resistantstarch**.com/ - 45k - Cached - Similar pages - Note this

[PDF] **Resistant Starch** – Questions and Answers
File Format: PDF/Adobe Acrobat - View as HTML
Resistant starch is generally considered to be represented by the total amount of **starch** and ... Therefore, **resistant starch** can be regarded as a component ...
www.nutrition.org.uk/upload/**Resistant**%20**Starch**(1).pdf - Similar pages - Note this

Result Page: 1 2 3 4 5 6 7 8 9 10 **Next**

Google resistant starch Search Advanced Search Preferences

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Web Results **1 - 10** of about **8,830** for **resistant starch**. (**0.12** seconds)

Health Report - 9/15/1997: **Resistant Starches**
Eating the recyclable package around your food as well as the food itself especially if it contains what are called probiotics Thats one of the dreams of Dr ...
www.abc.net.au/rn/talks/8.30/helthrpt/stories/s265.htm - 30k -
Cached - Similar pages - Note this

ABC Radio National - Health Report Transcript
Resistant starches are found in foods like rice, pasta, and potatoes. An Australian researcher who's been working in this field is Dr Jane Muir, ...
www.abc.net.au/rn/talks/8.30/helthrpt/hstories/hr140803.htm - 11k -
Cached - Similar pages - Note this

Resistance starch in the diet
In the 1980s a new type of naturally occurring dietary fibre was discovered and named **resistant starch**, as it "resists" digestion in the small intestine and ...
aww.ninemsn.com.au/article.aspx?id=50824 - 63k - Cached - Similar pages - Note this

Resistant starch puts fat on top of hit list
THE idea of burning more fat and boosting your metabolism without dramatically changing your diet sounds too good to be true, but the latest research shows ...
www.ferret.com.au/articles/z1/view.asp?id=99882 - 31k - Cached - Similar pages - Note this

Increasing **resistant starch** in grains to improve bowel health ...
The Food Futures Flagship is investigating the dietary benefits of **resistant starch** and developing commercial grain varieties that are high in **resistant** ...
www.csiro.au/science/ps201.html - 28k - Cached - Similar pages - Note this

Resistant starch: designing foods for better health (Publication ...
This two-page information sheet outlines the research programs in the Food Futures Flagship and Preventative Health Flagship to determine the health ...
www.csiro.au/resources/pff3.html - 22k - Cached - Similar pages - Note this
[More results from www.csiro.au]

Perth Diet Clinic : Consultant Dietitians and Nutritional Counsellors
Resistant starch is a type of **starch** that acts like dietary fibre. ... Indigestible fibre and **resistant starch** pass into the large intestine (large bowel) ...
www.perthdietclinic.com.au/article.asp?GroupID=32&ArticleID=185 - 10k -
Cached - Similar pages - Note this

Food Science and Nutrition: Food Science Australia
Resistant starch is found in many cereal grains, unripe fruits and some ... **Resistant starches** are defined as the **starch** that is not digested in the small ...
www.foodscience.csiro.au/fsn/1/fsn1e.htm - 12k - Cached - Similar pages - Note this

[PDF] Fact Sheet - **Resistant Starch**.pub
File Format: PDF/Adobe Acrobat - View as HTML
Resistant starch (RS) is regarded by many as a component. of dietary fibre. ... **Resistant starch** can be found naturally in foods such as ...
www.edgell.com.au/pdfs/Fact%20Sheet%20-%20**Resistant**%20**Starch**.pdf -
Similar pages - Note this

Diet and metabolic syndrome: where does **resistant starch** fit in?
This review examines the role **resistant starch** might play in the prevention and management of these conditions. Beginning with a definition of **resistant** ...
ro.uow.edu.au/hbspapers/41/ - 10k - Cached - Similar pages - Note this

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Resistant Starch – Questions and Answers

BRITISH Nutrition FOUNDATION

What is starch?

Starch is one of the main forms of carbohydrate in the diet. Starches are polysaccharides, i.e. they are made up of a number of glucose molecules which are linked together. They can therefore be described as complex carbohydrates. Starches are found in plant sources including potatoes and cereal products (e.g. bread, pasta). In general, digestible starches are broken down by digestive enzymes in the small intestine into glucose molecules. The glucose is then absorbed into the blood and used to provide energy for the body.

What is resistant starch?

Resistant starch is generally considered to be represented by the total amount of starch and the products of starch degradation that are not digested in the small intestine, and pass into the large intestine (or the colon). Therefore, resistant starch can be regarded as a component of dietary fibre.

Why is resistant starch not digested?

There are several reasons why resistant starch is not digested:

* The starch may be physically inaccessible to the digestive enzymes such as in grains, seeds or tubers.

* The starch granules themselves are structured in a way which prevents the digestive enzymes from breaking them down, e.g. raw potatoes and unripe bananas.

* When starches are heated they gelatinise and become more easily digested. However, if these starch gels are cooled, starch crystals form in the food that are resistant to enzyme digestion. This form of 'retrograded' starch is found in foods such as cornflakes or cooked and cooled potatoes.

* Starches that have been chemically treated (etherisation, esterisation, cross-bonding) cannot be broken down by digestive enzymes.

What are the sources of resistant starch in the diet?

Resistant starch is found in a wide range of foods including intact wholegrains, legumes, pasta, unripe bananas, raw potatoes, cooked and cooled potatoes, bread, cereals, some high fibre drinks and foods containing modified starches (some breads and cakes).

What happens to resistant starch in the gut?

Resistant starch reaches the large intestine (or the colon) virtually unchanged. However, it is then fermented by the microbial flora naturally present in the colon, to produce low levels of the gases carbon dioxide, methane and hydrogen. Additional fermentation products include organic acids and short chain fatty acids.

What are short chain fatty acids?

Short chain fatty acids (SCFA) are formed when polysaccharides are fermented by the anaerobic bacteria present in the large intestine. Many different forms of polysaccharides are present in the large intestine, including resistant starch. The main SCFAs produced in the human gut are butyrate, propionate and acetate. The concentrations of SCFAs in the large intestine vary depending on the types of polysaccharides although generally acetate is the most abundant and butyrate is the least abundant. Concentrations also vary in the different regions of the large intestine with higher concentrations detected in the area nearest the junction with the small intestine (70 – 140mM).

What effect do the short chain fatty acids have in the large intestine?

SCFAs lower the pH of the contents of the large intestine. This is of benefit to health because the reduced pH creates an environment that prevents the growth of harmful bacteria. A lower pH also aids in the absorption of minerals such as calcium and magnesium. SCFAs increase the blood flow to the colon and provide the cells in the wall of the intestine with a metabolic fuel (mainly in the form of butyrate). Additionally, butyrate has been shown to induce programmed cell death (apoptosis) and exert a level of control over the cell cycle. This suggests that butyrate might play an important role in maintaining the integrity of the gut wall by preventing the uncontrolled proliferation of abnormal cells that occurs in the early stages of colorectal cancer.

What are the health benefits of resistant starch?

Resistant starch contributes to the amount of fibre in the large bowel. Many health benefits have been attributed to foods providing resistant starch in the context of a high fibre diet. These include: a slower, more controlled release of glucose from the food into the blood stream (the glycaemic response – see below); improved bowel health; improved blood lipid profile; an increased feeling of satiety and increased micronutrient absorption (magnesium, calcium) in the colon. These factors may affect the risk of developing diseases such as colorectal cancer, cardiovascular disease, osteoporosis and obesity and assist in the management of diabetes, impaired glucose tolerance, inflammatory bowel diseases, diverticulosis and constipation.

How does resistant starch improve the glycaemic response after a meal?

When most carbohydrates are consumed, the levels of glucose in the blood are raised, peaking 15 – 45 minutes after finishing your meal. Levels return to normal within two to three hours. The concentration of the hormone insulin in the blood also increases in response to the elevated glucose concentration. High levels of insulin in the blood inhibit the use of stored body fat, as well as modulating appetite and satiety signals. Rapidly digestible carbohydrates,

such as those in white bread and confectionery, bring about a high glycaemic and insulinaemic response. In some instances, for example when you are being very active, this is of benefit as the glucose is rapidly absorbed into your blood stream to give you a fast sugar boost. However, carbohydrates that are broken down slowly, for example those from wholegrain foods or legumes, are generally better for keeping energy levels up throughout the day. This slower, more controlled glycaemic response is could be beneficial to all individuals as there is greater access to and use of stored fat, and hunger signals are suppressed. For people with diabetes or impaired glucose tolerance, increasing the amounts of resistant starch in the diet can help with the day-to-day management of blood glucose levels, as well as contributing to the body's use of fat.

Resistant starch has been shown to improve bowel health and function. How can this be assessed?

There are several markers that can be used to assess the health and function of the bowel. The time it takes for food to pass through the entire gut is referred to as the transit time. Healthy bowels process food quickly and gut cells absorb just enough water to produce firm stools. This, along with other factors, including the amount of fibre in the diet and the numbers of bacterial cells in the gut, affects the faecal weight. Some compounds, such as ammonia, phenols or bile acids are produced in the gut which can be damaging to the cells lining the large intestine. Low levels of these compounds in faeces suggest that the bowel is healthy and that their effects in the gut are lessened.

What are the improvements that can be seen in bowel health?

Resistant starch, like other forms of dietary fibre, helps to prevents constipation by providing bulk to the faeces. Bulky faeces move through the gut faster and result in an increased stool weight. Diets high in resistant starch have been shown to reduce the luminal pH, limiting the growth of harmful bacteria in the gut. They also decrease the amounts of secondary bile acids and ammonia present in the large bowel. Secondary bile acids can be converted into active compounds by the microbial flora and these can damage DNA. Ammonia has been shown to increase the proliferation rate of the cells in the gut wall. It is therefore advantageous to limit the concentrations of these compounds in the colon to reduce the risk of developing colorectal cancer. Additionally, the increased faecal bulk 'dilutes' the effect of any genotoxic agents in the large intestine, thereby reducing the extent of DNA damage to the cells lining the colon.

Resistant starch can function as a prebiotic. What does this mean?

Prebiotics are non-digestible food ingredients that stimulate the growth and activity of bacteria in the colon. Approximately 100 trillion bacteria live in our large intestines and they are essential for keeping our digestive system healthy. As resistant starch, along with other forms of dietary fibre, arrives in the large intestine unchanged, it provides metabolic substrates (fuel)

for these colonic bacteria and promotes the growth of the beneficial strains of bacteria (e.g. Bifidobacteria). A healthy bacterial flora further improves bowel health and function.

How can resistant starch be used to help prevent weight gain?

Due to it's indigestible nature, resistant starch has a lower calorific value than that of digestible starch (16kJ/g or 4kcal/g). Therefore, foods high in resistant starch may provide less energy weight for weight, although this will depend on the amounts of other nutrients in the food.

How much of a contribution should resistant starch make to the diet?

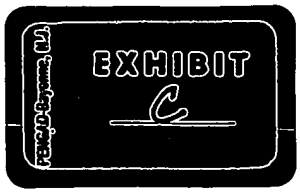
In the UK, resistant starch makes up a very small proportion of total daily starch intake. On average, the intake of all starches is 130g per day whereas the average intake of resistant starch is only around 4g per day. This is very low compared to countries in the developing world where intakes of resistant starch are much higher (for example 10g per day in India). Intake of dietary fibre in the UK should increase in order to improve bowel health and increasing consumption of foods rich in resistant starch is one way of achieving this. However, current intakes of dietary fibre in the UK are already well below recommendations and it has been recognised that substantial dietary changes would need to be made to reach these targets.

Is resistant starch safe?

Resistant starch is well tolerated in the diet. Minor effects of very high levels of resistant starch consumption are consistent with those of high fibre intakes, such as flatulence, belching, bloating and stomach aches which can occur when large amounts of resistant starch are fermented in the large intestine.

How can resistant starch be incorporated into meals?

You can up your intake of resistant starch, along with other forms of dietary fibre, by increasing the amount of wholegrain foods in your diet, for example seeded or granary breads, wholemeal pasta or high fibre cereals. There are also commercially produced resistant starches, for example Hi-maize™ or NOVELOSE 330™ which are sometimes used by food manufacturers to produce a food product that is high in fibre and which can also be lower in calories.



Re: Lactobacillus fermentum – US Patent application

(ix) Claim 7: "high amylase starch"

http://www.google.com.au/search?sourceid=navclient&ie=UTF-8&rls=GFRC,GFRC:2007-01,GFRC:en&q=high+amylose+starch

http://www.pharmcast.com/Patents/Yr2001/Sept2001/090401/6284273_Amylose090401.htm
http://www.google.com/patents?q=high+amylose+starch

http://www.ncbi.nlm.nih.gov/sites/entrez

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Results **1 - 10** of about **64,800** for **high amylose starch**. (**0.14** seconds)

Resistant **starch** - 2 visits - 3:16pm
This process, known as gelatinisation, makes the **starch** much more accessible to digestive enzymes. **Starch** with a **high amylose** content and **starch** which is ...
www.healthyeatingclub.com/info/articles/nutrients/resis**starch**.htm - 56k -
Cached - Similar pages - Note this

Starch
Of the two components of **starch**, **amylose** has the most useful functions as a hydrocolloid. Its extended conformation causes the **high** viscosity of ...
www.lsbu.ac.uk/water/hysta.html - 29k - Cached - Similar pages - Note this

Corn Fact Sheets: **High Amylose** Corn
It is grown exclusively for wet milling to produce a **starch** that crystallizes quickly. The **starch** from **high amylose** corn is used in textiles, gum candies, ...
web.aces.uiuc.edu/Value/factsheets/corn/fact-**amylose**.htm - 25k -
Cached - Similar pages - Note this

Specialty Corns: Waxy, **High-Amylose**, **High**-Oil, and **High**-Lysine ...
What are waxy, **high-amylose**, **high**-oil, and **high**-lysine corn types? WAXY CORN is a **starch** variant of normal corn. Waxy corn contains 100 percent amylopectin ...
ohioline.osu.edu/agf-fact/0112.html - 9k - Cached - Similar pages - Note this

New type of domestic film former — A **high amylose starch** product ... - 13 Dec
Further studies of the properties of the **high-amylose starch** products and creation of composites for treating glass fibres ...
www.springerlink.com/index/36321433563624R5.pdf - Similar pages - Note this

Preparation and Characteristics of **High-Amylose** Corn **Starch**/Pectin ...
RS-rich fractions can be obtained by hydrothermal treatments and retrogradation of native **high-amylose** corn **starch** (HACS).7 RS is made of short ...
www.aapspharmscitech.org/view.asp?art=pt060230 - 52k -
Cached - Similar pages - Note this

Antisense branches into **high amylose starch** - Nature Biotechnology - 13 Dec
In their paper on page 551, Schwall et al. use antisense technology to generate transgenic potatoes capable of producing the first truly **high-amylose** potato ...
www.nature.com/nbt/journal/v18/n5/full/nbt0500_481a.html - Similar pages - Note this

A **High Amylose** (Amylomaize) **Starch** Raises Proximal Large Bowel ... - 13 Dec
Final plasma cholesterol concentrations were significantly higher than initial values in pigs fed the 50/50 mixture of corn and **high amylose starch**. ...
jn.nutrition.org/cgi/content/abstract/127/4/615 - Similar pages - Note this

Imaging of **High-Amylose Starch** Tablets. 3. Initial Diffusion and ... - 13 Dec
The penetration of water into cross-linked **high amylose starch** tablets was studied at different temperatures by nuclear magnetic resonance (NMR) imaging, ...
pubs.acs.org/cgi-bin/abstract.cgi/bomaf6/2005/6/i06/abs/bm0503930.html -
Similar pages - Note this

Patent 6284273 - 2 visits - 13 Dec
Title: Cross-linked **high amylose starch** resistant to amylase as a matrix for ... The preferred cross-linked polymers of **high amylose starch** with covalent ...
www.pharmcast.com/Patents/Yr2001/Sept2001/090401/6284273_**Amylose**090401.htm -
28k - Cached - Similar pages - Note this

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Web

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[PDF] Plantic and National **Starch** Reach Agreement for **Starch** Supply and ...
File Format: PDF/Adobe Acrobat - View as HTML
a range of environmentally friendly plastics from naturally occurring **high amylose starch**, has entered into in an. agreement with National **Starch** and ...
www.plantic.com.au/docs/RNS_N**Starch**_051007.pdf - Similar pages - Note this

Plantic Technologies - **Starch** Technologies
Plantic's primary feedstock is a naturally **high amylose starch** (more than 70% **amylose**), derived from corn which has been hybridised over a number ...
www.plantic.com.au/our-technologies/capabilities/**starch**-technologies/ - 36k - Cached - Similar pages - Note this
[More results from www.plantic.com.au]

[PDF] New wheat promises dual benefits
File Format: PDF/Adobe Acrobat
has been carried out on **high-amylose starch**. from crop plants such as maize to give weight. C r o p p i n g. E x p e r i m e n t a l v a r i e t i e s ...
www.csiro.au/files/files/p857.pdf - Similar pages - Note this

Publications and presentations: Food Science Australia
Bajka B, Topping D, Cobiac L, Clarke J (2007) Butyrylated **High Amylose Starch** Is More Effective Than **High Amylose Starch** in Preventing Dietary ...
www.foodscience.csiro.au/records.htm - 16k - Cached - Similar pages - Note this

CSIRO SOLVE: WHEAT'S HEALTH KICK
High-amylose wheat (HAW) contains at least 70 per cent **amylose**, a resistant **starch** that the body finds harder to digest. **Starch** is a carbohydrate polymer ...
www.solve.csiro.au/0207/article5.htm - 17k - Cached - Similar pages - Note this

Gene silencing yields **high**-fibre wheat (ABC Science Online)
Usually, this enzyme would convert the **starch** molecule **amylose** to amylopectin. ... enzyme that needed to be silenced to give wheat with **high-amylose starch**. ...
www.abc.net.au/science/articles/2006/02/28/1576926.htm - 25k - Cached - Similar pages - Note this

Achieve Supplements Online - Discount Boydbuilding, Gym, Sports ...
... Vitis Vinifera Extract (Grape Seed), Water, Wazy Maize **Starch**, Wheat Flour, Wheat Protein Isolate, Whey ... Name, **High Amylose Starch** (Resistant **Starch**) ...
achievesupplements.com.au/atozdetails.php?word=**High+Amylose+Starch**+(Resistant+**Starch**) - 119k - Cached - Similar pages - Note this

Herbs
It is a comprehensive formulation consisting of soluble and insoluble fibres, including Psyllium Fibre, Soy Fibre, Resistant **Starch** (**High Amylose Starch**) ...
shopping.ninemsn.com.au/results/herbs/bcatid1441/forsale?text=category:herbs&page=4 - 130k - Cached - Similar pages - Note this

Innovation in Grain Production: Hi-maize® (Penford Australia)
In 1975, **Starch** Australasia commenced a research program to grow and develop uses for **high amylose** maize **starch**, particularly in foods. ...
www.gograins.com.au/grainsnutrition/ie/ie17_1.html - 10k - Cached - Similar pages - Note this

Food Science Project publications
A Lopez Rubio, A Htoon and E. Gilbert 'The Influence of Extrusion and Digestion on the Nanostructure of **High-Amylose Starches**. ...
www.ansto.gov.au/bragg/science/scientific_projects/food_science_project_publications.html

- 30k - Cached - Similar pages - Note this

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A High Amylose (Amylomaize) Starch Raises Proximal Large Bowel Starch and Increases Colon Length in Pigs[1,2,3]

David L. Topping,[4] James M. Gooden,[5] Ian L. Brown,[6] Debra A. Biebrick, Leanne McGrath, Rodney P. Trimble, Mingan Choct[7] and Richard J. Illman

CSIRO (Australia) Division of Human Nutrition, Adelaide 5000, Australia

ABSTRACT Young male pigs consumed a diet of fatty minced beef, safflower oil, skim milk powder, sucrose, cornstarch and wheat bran. Starch provided 50% of total daily energy either as low amylose cornstarch, high amylose (amylomaize) cornstarch or as a 50/50 mixture of corn and high amylose starch. Neither feed intake nor body weight gain as affected by dietary starch. Final plasma cholesterol concentrations were significantly higher than initial values in pigs fed the 50/50 mixture of corn and high amylose starch. Biliary concentrations of lithocholate and deoxycholate were lower in pigs fed high amylose starch. Large bowel length correlated positively with the dietary content of high amylose starch. Concentrations of butyrate in portal venous plasma were significantly lower in pigs fed high amylose starch than in those fed cornstarch. Neither large bowel digesta mass nor the concentrations of total or individual volatile fatty acids were affected by diet. However, the pool of propionate in the proximal colon and the concentration of propionate in feces were higher in pigs fed amylose starch. Concentrations of starch were uniformly low along the large bowel and were unaffected by starch type. In pigs with cecal cannula, digesta starch concentrations were higher with high amylose starch than with cornstarch. Electron micrographic examination of high amylose starch granules from these animals showed etching patterns similar to those of granules obtained from human ileostomy effluent. It appears that high amylose starch contributes to large bowel bacterial fermentation in the pig but that its utilization may be relatively rapid. J. Nutr. 127: 615–622, 1997.

KEY WORDS: • *pigs* • *volatile fatty acids* • *large bowel* • *starch* • *steroid metabolism*

The fermentation of carbohydrates by human large bowel microflora is a focus of considerable interest. In large part, this is due to recognition that the resulting volatile fatty acids (VFA)[8] contribute substantially to the health and integrity of the colon (Cummings and Macfarlane 1991). In addition to general effects such as lowering of colonic pH and prevention of diarrhea, two of the three major VFA (acetate, propionate and butyrate) appear to have specific benefits. Propionate enhances colonic muscular contraction in vitro (Yajima 1985) and also may promote large bowel blood flow through relaxation of the vasculature (Mortensen et al. 1991). Butyrate is probably even more important for the maintenance of colonic health. This acid is recognized as a major metabolic fuel for normal colonocytes (Roediger 1982), and its infusion promptly relieves ulcerative colitis (Scheppach et al. 1992). Butyrate also assists in the maintenance of a normal cell phenotype through a number of mechanisms that involve the repair of damaged DNA and suppression of the growth of transformed cells (Kruh et al. 1994).

Factors that control VFA production are of some significance and, until relatively recently, it was thought that nonstarch polysaccharides (NSP, major components of dietary fiber) were the principal fermentative substrates for the colonic microflora. This is not surprising, given the resistance of NSP to the digestive action of human gastrointestinal enzymes and the assumption that starch, the major polysaccharide in humans diets, is digested completely in the human small intestine. Now, there is evidence that a considerable fraction of dietary starch enters the human large bowel, undigested (Cummings and Macfarlane 1991). This fraction is termed resistant starch (RS) through its resistance to amylase degradation. In the large bowel, RS is fermented by the microflora, and data from in vitro fermentation studies with human fecal inocula suggest that starch fermentation may be especially useful because it favors the production of butyrate (Weaver et al. 1992).

Resistant starch offers an opportunity to increase the fermentable polysaccharide content of carbohydrate-based foods

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[4] To whom correspondence should be addressed.

[5] Present address: Department of Animal Science, University of Sydney, Camden NSW 2570, Australia.

[6] Present address: Goodman Fielder Ingredients Ltd., 42-45 Green Street, Botany, NSW 2019, Australia.

[7] Present address: Monogastric Unit, Department of Animal Science, University of New England, Armidale, NSW 2351, Australia.

[8] Abbreviations used: C, cornstarch diet; CHA, corn and high amylose starch diet; HA, high amylose starch diet; NSP, non-starch polysaccharides ("fiber"); PEG, polyethylene glycol; RS, resistant starch; TAG, triacylglycerol; VFA, volatile fatty acids.

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without necessarily altering their organoleptic properties. However, in marked contrast to NSP (in which resistance to human enzymic digestion is a matter of polymer structure), RS occurs for a number of chemical, technological and physiological reasons (Annison and Topping 1994). This means that the RS content of a food, measured as resistance to enzymic hydrolysis in vitro, need not reflect the starch in the food that resists amylolysis in vivo. From the standpoint of food manufacture, there is a clear advantage in the availability of starch types for which the RS content of the starting material is known and is retained in the food product through the human small intestine. Studies in humans have shown that with one such starch (Hylon VII), there was a significant increase in breath H_2 evolution and the excretion of total and individual VFA (van Munster et al. 1994). High amylose starches are found in a variety of cereal cultivars and offer another opportunity to modify the RS content of foods. The crystalline structure of amylose means that it gelatinizes less readily than amylopectin when heated with water so that with appropriate processing, foods containing high amylose starches can be enriched in RS. High amylose starches obtained from corn (often referred to as amylomaize starches) seem to be useful in this regard. Studies in humans with ileostomy have shown a significant excretion of starch when products containing one such high amylose starch are consumed (Muir et al. 1994). These authors showed also that the "dietary fiber" content of this starch was ~30% on analysis.

Although there is support for greater large bowel fermentation with consumption of RS, there are data indicating that this does not necessarily increase fecal VFA excretion (Tomlin and Read 1990). Thus, there is a degree of uncertainty about the change in colonic VFA following RS ingestion. Investigating changes in the proximal human colon in situ is difficult for a number of reasons including ethical and anatomical considerations. The pig is considered to be a good model for human fiber metabolism (Graham and Aman 1982) and provides a useful way of probing effects of such dietary treatment on large bowel VFA. Studies in pigs fed different foods varying in fat and NSP have shown that VFA determined in the distal colon need not reflect those in the proximal large bowel (Topping et al. 1993), so that concentrations could be raised in the proximal colon with little or no change in the distal region or in feces. We decided to compare the effects of a high amylose (RS) and high amylopectin starch (both from corn) on the distribution of VFA and related variables throughout the colon in pigs fed a diet based on human foods. This appears to be the first time that such a study has been conducted. Because pigs resemble humans also in their responses in plasma lipids following dietary change (Siebert et al. 1987), we examined effects on plasma lipids. In view of the fact that dietary RS alters fecal bile acids (van Munster et al. 1994), biliary bile acids were measured as well to determine whether there was any effect on the enterohepatic circulation of bile acids.

MATERIALS AND METHODS

Animals. Young adult male pigs of the Large White strain were used. All of the animals were purchased from a commercial piggery (Millwards' Piggery, Eudunda, SA, Australia) and were ~14 wk old at the start of the experiment. The animals were housed in individual pens and fed a standard pig production diet until the experiments commenced (Topping et al. 1993). All of the procedures described were approved formally by the Animal Care and Ethics Committees of Division of Human Nutrition and conformed to published guidelines (National Health and Medical Research Council, CSIRO and Australian Agricultural Council 1985).

Dietary study

Diets and feeding procedures. Twenty-four pigs were used for this experiment and were fed a diet comprised of commercially available human foods that has been described previously (Topping et al. 1993). In brief, the diet was formulated from minced beef, corn oil and cornstarch with 50 g of NSP/d provided as a wheat bran product (All Bran, Kellogg Australia, Pagewood, NSW, Australia). Allowance was made for the fat, protein and carbohydrate in the latter product. The diet provided 37.5% of energy as fat, 50% of energy as carbohydrate (of which 48.2% was as starch) and 12.5% of energy as protein. A commercial vitamin and mineral supplement was also added to the diet (Topping et al. 1993). The starch, corn oil, wheat bran, vitamins and minerals were mixed and pelleted and then weighed into individual meals. The minced beef was frozen in individual portions and thawed overnight before mixing with the pellets and feeding to the animals. There were three experimental groups, each consisting of eight pigs. One group was fed the diet with all of the cornstarch as a low amylose starch (diet C). In the second group, 56% of the starch (or 28% of total energy) was fed as cornstarch and the remainder as a high amylose starch (Hi-maize™, Starch Australasia, Botany, NSW, Australia; diet CHA). The third group of pigs was fed the diet containing 94% of starch as high amylose starch (the remainder of the starch being in the wheat bran; diet HA). The pigs were fed one meal daily (at 0900–1000 h) with sufficient food to provide an intake of 16 MJ/d. They were allowed free access to water. On the day before sampling the pigs were given 5 g of polyethylene glycol (PEG) in 200 mL of water with the meal as a marker of fluid phase transit (Malawer and Powell 1967).

Sampling procedures. The pigs were fed the experimental diets for 3 wk. After 5 d of feeding, fresh feces were collected at approximately 1000 h from the pigs fed the low and high amylose diets. These samples were analyzed for VFA concentrations. At the end of the 3-wk feeding period, the pigs were sampled as described previously (Topping et al. 1993). In brief, food was withdrawn on the morning of sampling and the pigs were sedated with ketamine (Ketapex; Apex Laboratories, St Mary's, NSW, Australia) and then anesthetized with halothane in O_2. After a midline laparotomy, the stomach and intestines were retracted to expose the hepatic portal vein. Blood was drawn from that vessel by syringe, collected into ice-cold tubes containing EDTA as anticoagulant; plasma was prepared by centrifugation at 3000 × *g* for 10 min and stored at −20°C prior to analysis. The gall bladder was identified and drained of bile by needle and syringe. The volume of bile was measured and a portion frozen and stored for analysis of bile acids and neutral sterols. The esophagus and the rectum were ligated and the whole gut excised. The cecum was tied off from the terminal ileum, the whole large bowel was separated from the mesentery and laid out to approximately the same tension and its total length measured; then the colon was subdivided into five sections of equal length which were isolated with ligatures (starting at the proximal region). These sections were numbered 1–5 from the proximal colon and their contents and that of the cecum were extruded and weighed. The first animal was anesthetized at 0830 h and sampled at 0900 h; each pig took ~40 min to process. Six pigs were sampled daily and the same sequence was maintained throughout the sampling period; thus, a pig from each group was sampled at 0900, 0945, 1030, 1115, 1200 and 1245 h. One animal (fed high amylose starch) suffered damage to a leg 5 d before the end of the experiment and required analgesics. This pig was sampled with the others to maintain the balance of the feeding and sampling procedures but the data were not used for statistical evaluation.

Cecostomized pigs

Surgical preparation of animals. Four pigs were fitted with a cannula in the cecum to allow continuous sampling of gut contents. After overnight food deprivation, the pigs were sedated as described above. When they were unconscious, the hair was removed from around the surgical site. A 10-cm incision was made in the right flank behind the last rib, and the large bowel was exposed carefully. The cecum was located and a small incision made in its extremity. A 500-mm silastic tube (13 mm o.d., 8 mm i.d.) was inserted through this hole to a depth of ~90 mm. The tube was tied in place with

purse-string sutures on either side of two plastic cuffs cemented to the tube. To add extra support, a disc of high density polyethylene (diameter 45 mm, thickness 1.5 mm) was slipped down the tube and sutured to the cecum. The cannula was exteriorized through a stab wound, dorsal to the incision, anchored to the skin with nonabsorbable sutures and sealed with a removable plastic plug. Two milliliters of a 5 g/L solution of bupivacaine hydrochloride, (Marcain; Astra Pharmaceuticals, North Ryde, NSW, Australia) was used as a postoperative nerve block for pain relief on the dorsal side of the incision. Recovery from surgery was rapid; pigs returned to normal food intake within 2–3 d during which time they were maintained on standard ration.

Feeding and sampling procedures. During the recovery period the pigs were fed once daily at 900–1000 h. To determine starch concentrations in cecal digesta, two pigs were fed a single meal of the diet containing high amylose starch and two were fed a meal of the low amylose starch diet at 0930 h. Samples were taken from the cecum at 1300, 1500, and 1700 h and transferred promptly to a chilled centrifuge tube. Sampling was achieved by passing a Ryles tube (FG 18, Adelaide Surgical Supply, Somerton Park, SA, Australia) down the cannula into the cecum and drawing up 10 mL of contents with a syringe using gentle suction.

Analytical procedures

Concentrations of total cholesterol, VFA in portal venous plasma and digesta, and bile acids and neutral sterols in bile were determined by gas-liquid chromatography as described previously (Topping et al. 1993). Digesta from each region of the large bowel of intact animals were extruded, diluted with 2–3 volumes of water and aliquots taken for measurement of VFA (Topping et al. 1993). A similar procedure was used for the measurement of VFA in fresh feces. PEG was determined spectrophotometrically in digesta samples, and starch was measured by determination of free glucose using a commercial kit (Boehringer Mannheim, Germany) after amylase (Sigma, St Louis, MO) and amyloglucosidase digestion (Boehringer Mannheim). To ensure complete recovery of starch, samples were dispersed in dimethylsulfoxide. Samples from cannulated pigs were freeze-dried for determination of total starch. Portions of the freeze-dried samples were sprinkled carefully onto double-sided adhesive tape attached to an aluminium stub (Lineback and Ponpipom 1997). The samples were coated thinly with gold (4 min at 20 mA) using a Polaron E5100 Sputter Coater (Polaron Equipment, Watford, Hertfordshire, U.K.) and viewed under electron microcopy (JSM-35-35, Japan Electron Optics Laboratory, Nakagami, Akishima, Japan). These images were recorded on black and white photographic film. Samples of native amylomaize starch, amylomaize starch after incubation with thermostable bacterial α-amylase and pullulanase were processed; in vitro and high amylose starch granules recovered from human ileostomy effluent were processed in the same way.

Statistical methods. Data for intact animals are shown as the means for eight observations per group with the exception of the high amylose group (7 pigs) with a pooled standard error of the mean. The statistical significance of differences between treatments was established by ANOVA incorporating a test for least significant difference using a computer (AMR, Adelaide, SA, Australia). Comparison of initial and final concentrations of plasma lipids was conducted using a repeated measures ANOVA. Effects of diet and colonic region on the distribution of starch and VFA along the large bowel were analyzed by ANOVA (Genstat 1988) using a Sun Sparc Station (Sun Microsystems, Sydney, NSW). Simple correlations between variables were calculated by linear regression analysis. A value of $P < 0.05$ was taken as the criterion of significance. Individual data are shown for the starch concentrations in cannulated animals.

RESULTS

Dietary adaptation to high amylose starch

Food consumption and body weight gain. At the start of the experiment the pigs were allocated randomly to each group and the mean body weight (all groups combined) was 44 kg (pooled SEM = 2, $n = 24$). The pigs found the diets palatable and all food was consumed promptly after presentation. At the end of the experiment, there were no differences in body weight with means of 53 (group C), 52 (group CHA) and 52 kg (group HA) (pooled SEM = 2).

Plasma lipids. Plasma cholesterol concentrations at the start of the experiment did not differ among groups with means of 1.88, 1.93 and 1.87 mmol/L in groups C, CHA and HA, starch, respectively (pooled SEM = 0.10, $n = 24$). Initial plasma cholesterol was a significant covariate ($P < 0.001$) with an apparently linear relationship between initial and final values. Final values (adjusted for initial concentrations) were 2.05, 2.25 and 1.92 mmol/L (SEM of the difference = 0.15). Only in pigs fed the CHA diet were final plasma cholesterol concentrations significantly ($P < 0.02$) higher than the initial value.

Plasma triacylglycerol (TAG) concentrations also were not distributed normally and an appropriate distribution was achieved on conversion to $\log_{10}$. In contrast to plasma cholesterol, initial values were not a significant covariate and final concentrations did not differ among groups with a combined mean of 0.12 mmol/L (pooled SEM = 0.02, $n = 23$).

Biliary bile acids and cholesterol. The volume of gall bladder bile was unaffected by dietary treatment with mean values of 65, 63 and 58 mL (pooled SEM = 8) for pigs fed the C, CHA and HA diets, respectively. Concentrations of individual bile acids are shown in **Table 1.** The major acids were unaffected by dietary treatment, but concentrations of lithocholate and deoxycholate were lower in pigs fed the CHA and HA diets than in those fed the C diet. The pools (i.e., concentration × volume) of biliary cholesterol and total bile acids were unaffected by diet. Mean values for the former were C group, 0.24 mmol; CHA group, starch, 0.21 mmol; and HA group, 0.29 mmol (pooled SEM = 0.06). Corresponding values for total bile acids were 4.1, 3.6 and 4.9 mmol (pooled SEM = 1.0).

Portal venous VFA. Concentrations of total and individual VFA did not differ between the pigs fed the C and CHA diets (**Table 2**). However, concentrations of total VFA and of acetate were significantly lower in pigs fed the HA diet than in the other two groups. Concentrations of propionate in pigs fed the mixture did not differ from either of the other two groups. Butyrate concentrations in pigs fed the HA diet were significantly lower than in those fed diet C.

Fecal VFA and large bowel length, digesta, PEG and VFA. Concentrations in fresh feces were determined in pigs fed the HA and C diets. Concentrations of acetate, butyrate and total VFA were unaffected by dietary treatment but propionate was significantly higher in pigs fed the HA diet than in those fed the C diet (**Table 3**).

Large bowel length was affected by diet and was significantly greater in pigs fed the HA diet than in those fed the C diet. Colon length in pigs fed the mixture did not differ significantly from those fed the other two diets. There was a linear relationship between the mean length of the colon and the proportion of dietary starch as high amylose starch (**Fig. 1,** $r = 0.985$, $P < 0.02$).

The mass of total digesta was unaffected by diet with mean values of 627 (C), 652 (CHA) and 720 g (HA; pooled SEM = 115). There was no difference in digesta mass among any of the groups at any sampling site. Digesta mass was highest in the proximal colon and lowest in the distal colon (data not shown).

Concentrations of total and individual major VFA were unaffected by dietary treatment at any sampling site along the large bowel (data not shown). Similarly, the pools of total VFA in the whole large bowel were unaffected by diet with

TABLE 1

Concentrations of individual bile acids in gall bladder bile of pigs fed diets containing cornstarch (C), high amylose (amylomaize) starch (HA) or a mixture of corn and high amylose starch (CHA)[1]

Diet	*n*	Lithocholate	Deoxycholate	Chenodeoxycholate	Hyodeoxycholate	Hyocholate
				mmol/L		
C	8	0.2[a]	0.5[a]	16.0	43.7	6.1
CHA	8	0[b]	0.1[b]	16.6	36.0	8.3
HA	7	0[b]	0.1[b]	17.5	45.0	8.8
Pooled SEM		0.1	0.1	2.2	4.5	3.0

[1] Data are shown as the mean and pooled SEM. Means in a column not sharing a superscript are significantly different ($P < 0.05$).

mean values of 37.2, 43.8 and 42.8 mmol (pooled SEM = 9.7) for the C, CHA and HA groups, respectively. As might be expected from the data for total VFA, there were no differences among treatment groups in the pools of the individual VFA. Acetate pools averaged 20.7 (C), 22.2 (CHA) and 21.4 mmol (HA; pooled SEM = 5.9). Corresponding values were 9.3, 13.5 and 11.9 mmol (pooled SEM = 3.7) for propionate and 4.0, 3.9 and 4.4 mmol (pooled SEM = 0.8) for butyrate. The distribution of the major VFA paralleled that in digesta, and there were no effects of treatment on acetate and butyrate at any sampling site when the data were examined untransformed or as logarithms. However, it was clear that in the case of propionate, the data were not distributed normally. On logarithmic transformation, there was an effect of treatment in the cecum and colon section 1 with the pools being significantly higher in pigs fed the HA diet (1.7 mmol, cecum; 4.3 mmol, colon 1) than in those fed the C diet (0.8 mmol, cecum; 2.1 mmol, colon 1). Corresponding values for pigs fed the CHA diet were 1.1 and 5.6 mmol and these did not differ from the other two groups.

PEG pools were unaffected by dietary treatment at any sampling site and followed closely the distribution profile of the mass of digesta (data not shown). The total recovery of the administered dose was 67–69%.

Large bowel starch. The concentrations and pools (<0.5% of that fed) of starch along the large bowel were uniformly low and were unaffected by dietary treatment (data not shown).

Studies in cannulated pigs

Starch in cecal digesta. In pigs fed the standard pig ration, concentrations of starch in cecal digesta were low with mean values of 1.8 (±0.6), 1.8 (±1.0) and 1.8 (±0.8) mg/g of wet weight (mean ± SEM, $n = 4$). These samples were taken at the same times as those at which the pigs were fed the test meals (i.e., 3.5, 5.5 and 7.5 h after feeding). In pigs fed those test meals containing cornstarch, concentrations were low at all sampling times in one animal and somewhat higher in the other (**Table 4**). Concentrations were much higher in pigs fed the HA diet although at 7.5 h after feeding, it was not possible to obtain a sample from one animal as the cecum appeared to be empty. Samples could not be drawn from the cecum on the following morning.

A portion of the digesta recovered from the cecum was examined using a scanning electron microscope. The starch granule remnants found in these samples (**Fig. 2*a***) were quite unlike native high amylose cornstarch (Fig. 2*b*). However, they did resemble high amylose starch granules following amylolysis in vitro (Fig. 2*c*) or in ileostomy effluent from an individual who had eaten a bread containing high amylose starch (Fig. 2*d*). The common features of all of these samples were considerable surface erosion and pitting of the granules.

TABLE 2

Concentrations of total and individual volatile fatty acids in portal venous plasma of pigs fed diets containing cornstarch (C), high amylose (amylomaize) starch (HA) or a mixture of corn and high amylose starch (CHA)[1]

Diet	*n*	Acetate	Propionate	Butyrate	Total
			mmol/L		
C	8	0.41[a]	0.20	0.10[a]	0.71[a]
CHA	8	0.43[a]	0.20	0.08[a,b]	0.71[a]
HA	1	0.30[b]	0.16	0.05[b]	0.51[b]
Pooled SEM		0.04	0.03	0.01	0.08

[1] Data are shown as the mean and pooled SEM. Means in a column not sharing a superscript are significantly different ($P < 0.05$).

DISCUSSION

In these experiments we have examined the effects of the consumption of a high amylose starch on a number of important variables in pigs. These variables include large bowel and fecal VFA, large bowel starch and plasma lipids and biliary steroids, and all have implications for human health.

One of the potentially important effects of high amylose starches is lowering of plasma cholesterol concentrations with the attendant reduction in the risk of coronary disease. Soluble NSP lower plasma cholesterol in part through enhanced fecal bile acid and neutral sterol excretion, which appear to be effected through changes in the viscosity of small intestinal

TABLE 3

Concentrations of total and individual volatile fatty acids in feces of pigs after consuming diets containing cornstarch (C) or high amylose (amylomaize) starch (HA) for 5[1]

Diet	*n*	Acetate	Propionate	Butyrate	Total
			mmol/L		
C	8	47.8	22.1[b]	7.4	85.4
HA	7	63.9	47.8[a]	13.7	126.9
Pooled SEM		14.6	8.4	3.1	16.3

[1] Data are shown as the mean and pooled SEM. Means with unlike superscripts are significantly different ($P < 0.05$).

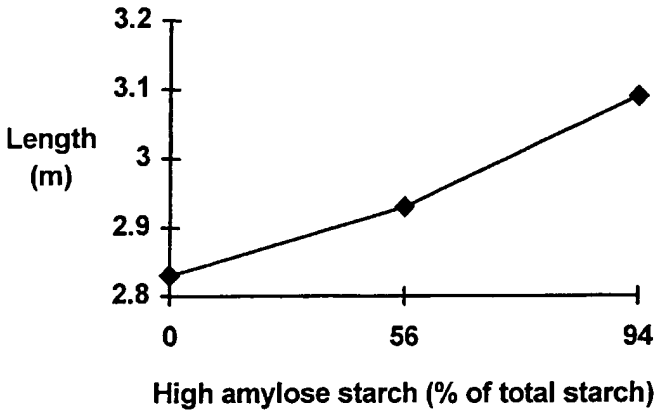

FIGURE 1 Colon length vs. dietary content of high amylose cornstarch in pigs. Data are shown as the means for each group (pooled SEM = 0.08).

contents (Gallaher et al. 1993). Given the physicochemical properties of high amylose cornstarches (which can increase the viscosity of dispersions), it might have been possible that they resemble soluble NSP (Brown 1993). Further, it has been reported that undigested starches from complexes with bile acids which might increase their fecal excretion (Abadie et al. 1994). These possibilities have been supported with a reduction of plasma cholesterol and increased lipoprotein catabolism in rats fed a hypercholesterolemic diet containing a high amylose starch (Mazur et al. 1990). However, another study in rats failed to show any change in plasma cholesterol although plasma triacylglycerols were significantly lower (Goda et al. 1994). In the present experiment, there was no effect of starch type on either plasma cholesterol or total biliary steroids. Indeed, in pigs fed the mixture of starches, total plasma cholesterol was significantly higher than in those fed high amylose starch—a finding for which we have no explanation. The lack of a clear effect of dietary level of high amylose starch on plasma cholesterol and biliary steroids in animals fed human foods in the present study suggests that it is unlikely to be a major agent for lowering plasma cholesterol. This conclusion supports recent studies in humans by Noakes et al. (1996) who used the same high amylose starch as that used in the present study. The lack of effect may be explained by the fact that high amylose starches form viscous solutions only when gelatinized fully (Brown 1993). This could occur during the production of rat diets.

Secondary bile acids arise through the bacterial metabolism of primary bile acids in the large bowel. There was no effect of diet on hyodeoxycholate (which is the major secondary bile acid in the pig), but concentrations of two minor secondary acids (lithocholate and deoxycholate) were lower in animals fed high amylose starch. This suggests a possible change in the microflora. Lithocholate and deoxycholate were found in only 3 of the 15 pigs fed the high amylose diets as opposed to 7 of the 8 fed the control diet. Lower fecal concentrations of deoxycholate and a greater proportion of primary acids have been reported in humans eating a diet enriched with RS (van Munster et al. 1994). Secondary bile acids are thought to be mutagenic, and these data were interpreted as showing reduced risk of colonic carcinogenesis because there was also a fall in the cytotoxicity of fecal water. Our data support a role for RS in modifying large bowel bile acid metabolism and show for the first time that this involves the recycling of secondary bile acids to the gall bladder. It should be noted that although we did not measure the RS content of the high amylose starch used in the present study, it was the same as that used by Muir et al. (1994). Their data suggested that 30% of the starch would resist digestion by human gut enzymes. Coupled with the high concentrations of starch in the cecum of cannulated pigs, this justifies the description of this starch as RS.

No differences were found in plasma triacylglycerols among any of the treatments. This finding stands in contrast to other data in rats where it has been shown that a diet containing high amylose starch lowered hepatic lipogenesis and plasma total (Goda et al. 1994) and VLDL triacylglycerols (Mazur et al. 1990). Lipogenesis is a major determinant of hepatic VLDL secretion (Windmueller and Spaeth 1967), so that the lower plasma concentrations are consistent with diminished production. The reason for the difference between the present data and those published in the rat may be due to species differences in lipoprotein production. In the pig plasma, triacylglycerol concentrations are relatively low, partially as a consequence of hepatic secretion of a triacylglycerol-poor intermediate density lipoprotein (IDL) (Huff et al. 1985). Thus, altered hepatic fatty acid synthesis might not limit triacylglycerol secretion as much as in species such as the rat in which triacylglycerol-rich particles are the main lipoprotein exported by the liver.

The feeding of high amylose starch raised both the concentration and relative contribution of propionate in fresh feces. It was not possible in this experiment to make total fecal collections, thus, absolute excretion rates cannot be calculated. However, the change in propionate is consistent with previous data from pigs showing that increased RS (as legumes) raised large bowel propionate (Fleming et al. 1989, Topping et al. 1993). However, they conflict with other data from the same species which showed that rice raised butyrate excretion (Marsono et al. 1993), suggesting that the type of starch affects the VFA which are excreted.

As in human studies, the pigs were not fed for some time before sampling to avoid any effect of meal passage on plasma and biliary steroids. This approach has been used in earlier experiments in which the sources and dietary concentrations of NSP and starch were varied and very substantial differences were noted in the masses of large bowel digesta and the concentration and pools of VFA (Marsono et al. 1993, Topping et al. 1993). In those experiments, some foods (e.g., navy beans, brown rice) gave much greater digesta and VFA masses than would be predicted from their NSP content—a difference that may be explained through the presence of resistant starch. In marked contrast, there were no effects of dietary inclusion of high amylose starch on total digesta or VFA in this experi-

TABLE 4

Concentrations of starch in cecal digesta of pigs with cecal cannula fed diets containing either cornstarch (C) or a high amylose (amylomaize) starch (HA)[1]

	Time after feeding (h)		
Diet	3.5	5.5	7.5
	mg/g dry matter		
C			
Pig 1	1.1	2.2	0.8
Pig 2	15.7	5.5	8.6
HA			
Pig 3	29.6	65.9	No sample
Pig 4	48.6	50.1	71.0

[1] Data are individual values for each pig.

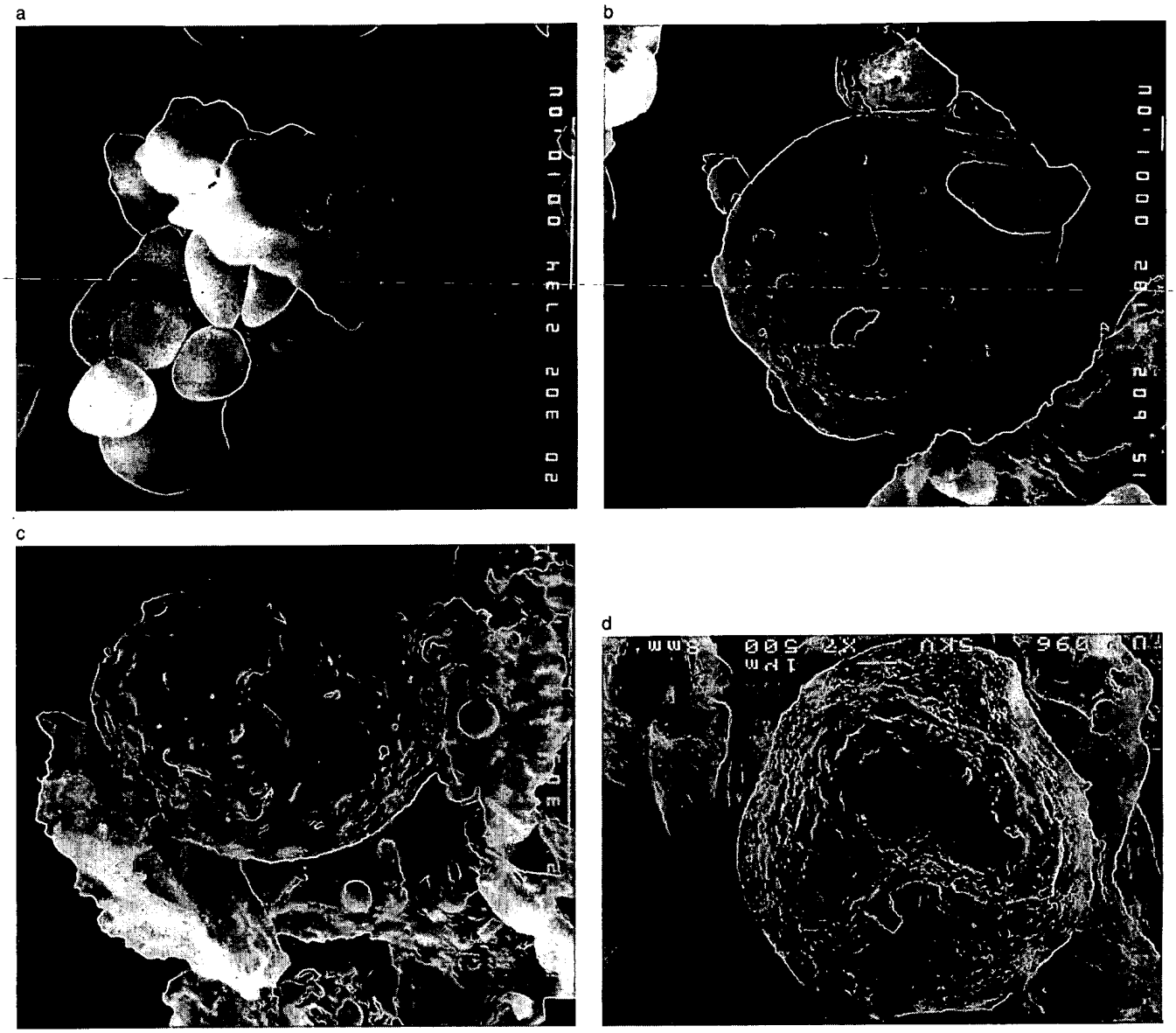

FIGURE 2 Electron micrographs of *a*) native high amylose starch, ×3000; *b*) high amylose starch after digestion with thermostable bacterial α-amylase and pullulanase, ×6000; *c*) high amylose starch granules recovered from pig cecum, ×4800; and *d*) high amylose starch granules recovered from human ileostomy effluent, ×7500.

ment. Concentrations of starch in the large bowel were also extremely low. The lack of effect of dietary treatment, coupled with the very low starch concentrations in the large bowel, suggested that the character of the starch was altered by the means of incorporation into the diet so as to render it more susceptible to amylolysis in the small intestine. This possibility may be discounted by the observations of shifts in propionate excretion in intact animals and the fact that cecal starch levels were much higher in cannulated pigs fed high amylose starch than in animals fed low amylose starch (although it must be noted that in the latter, starch was detected in digesta).

These data from the cannulated pigs provide an explanation also for the absence of any differences in colonic VFA and for inconsistencies which have been noted among some of the studies in humans. In both pigs fed high amylose starch, cecal starch was high at 5 h after feeding. This is also the approximate time of the appearance of other starchy food such as beans (Fleming et al. 1989) and rice in the large bowel of cannulated pigs. Digesta samples could be drawn from all pigs with little or no difficulty 5 h after feeding, but at 9 h, the cecum of one animal fed high amylose starch appeared to be empty and sampling was not possible the next morning. These data are different from those obtained in similar studies with rice in which the concentration of starch in cecal digesta as raised for at least 14 h after feeding (Marsono, Y., Davies, D. A., Illman, R. J., Gooden, J. M. and Topping, D. L., unpublished observations). It appears that either the transit of high amylose starch is particularly rapid or that it was fermented very quickly compared with other foods and that the VFA may be absorbed in the proximal colon. The first choice is not supported by the similarity of distribution of PEG between the groups, whereas rapid fermentation is supported by the low starch levels in colonic digesta in the present studies. Complete fermentation in the proximal colon is consistent also

with the data of Tomlin and Read (1990) obtained from intact humans eating RS as cornflakes. In these volunteers, there was an increase in colonic fermentative activity as measured through breath H_2 evolution but no change in fecal parameters. The rise in breath H_2 occurred 1–2 h after feeding and returned to base-line values within several hours. Therefore, it seems that RS is fermented relatively quickly and that the resulting VFA are absorbed in the proximal colon leaving fecal parameters relatively unchanged. This suggestion would account for the rather small differences in fecal bulk seen by van Munster et al. (1994) in humans fed relatively large quantities of RS. If so, then it has important implications for physiological effects of RS because most degenerative large bowel disease is found in the distal colon. In terms of experimental design, it may be especially important, with measurement of colonic VFA taking place a relatively short time after feeding. It is equally possible that in the present experiment there was another influence on the speed of fermentation, i.e., the presence of wheat bran. NSP mixtures are fermented rather more effectively in the large bowel than are single polysaccharides (Storer et al. 1984) so that fermentation might have been slower if the diet contained no additional fiber.

The pig is considered a good model for human fiber metabolism and large bowel physiology (Graham and Aman 1982), and the present electron microscopic data show that this is true also for starch digestion. Native high amylose cornstarch granules exhibit rounded or polyhedral shapes with some having an irregular or filamentous appearance (Sandstedt 1965). The degree of irregularity increases with the amount of amylose in the granule (Wolf et al. 1964). Exposure in vitro of these starch granules to amylolysis by bacterial α-amylase and pullulanase produces a pattern of surface erosion often resulting in a pit that extends to a hollow core. This seems to be due to the fact that the core regions are more susceptible to hydrolysis, possibly because of conformational differences between the core and the outer regions. Similar erosion patterns were observed in the granular material recovered from cecal digesta of pigs fed the amylomaize starch. These patterns occur through the sequential actions of gut and bacterial amylases. The electron micrographs show great similarity between the granules recovered from the pig cecum and those from the effluent of human ileostomates who had consumed high amylose starch (Muir et al. 1994).

Analysis of the distribution of VFA and digesta along the porcine large bowel shows high concentrations and pools of VFA and digesta masses in the proximal colon with a decrease towards the distal region (Bach-Knudsen et al. 1993, Marsono et al. 1993, Topping et al. 1993). A similar profile was noted in the present experiment and is consistent with higher fermentative activity (through greater substrate availability) in the proximal large bowel. Previous studies have shown that the exact distribution of these variables cannot be predicted from their values in the distal colon (Topping et al. 1993). The same was true in the present experiments in that propionate was not distributed normally. Although there were no overall differences in VFA and digesta, concentrations and pools of propionate in the proximal large bowel of animals fed the high amylose starch were greater. These data are consistent with fecal propionate excretion and with data from previous studies with beans in which it was shown that in the proximal large bowel the molar proportion of butyrate was lower (Fleming et al. 1989) and the absolute quantities of propionate were greater (Topping et al. 1993).

Studies in rats have shown that one of the consequences of increased dietary intake of fermentable carbohydrates is an increase in the volume and weight of the cecum and its contents (e.g., Goodlad and Mathers 1990). These increases are accompanied by histological changes in the gut wall with increases in crypt numbers and their depth. Similar results have been obtained in pigs whose adaptation to dietary NSP increased the mass of the colon (Pond and Varel 1989); it is thought that these trophic effects were mediated (at least in part) by VFA. The present data show that there appears to be a dose-dependence in the lengthening in the large bowel of pigs fed high amylose starch. The implications for colonic health are not entirely certain but may be of benefit in preventing degenerative bowel disease. Greater colon length has been noted in rats fed this high amylose starch, whereas in other animals with experimentally induced colitis, the injured area was reduced (Ikai, M., Morita, T. and Kiriyama, S., Yamanouchi Pharmaceutical, personal communication). It is possible that the greater length of the bowel could have provided an explanation for the lower concentrations of butyrate in portal venous plasma. Because butyrate is believed to be a preferred substrate for colonocytes, it may be presumed that greater cell numbers would mean more demand for this substrate and, hence, less butyrate available for transport via the portal vein. However, this is not the case because linear regression analysis showed no relationship between colon length and portal venous butyrate ($r = -0.19$, $P > 0.0.5$).

Although the bacterial fermentation of resistant starch in the colon recovers metabolizable energy for the host, it is intrinsically less efficient than complete digestion and absorption in the small intestine. The reasons for the relative inefficiency of microbial metabolism include retention of energy by the bacteria and unavoidable losses as heat and gases (Livesey 1990). It appears that ~7.6 kJ/g starch might be available as VFA produced through RS fermentation compared with 16.4 kJ/g from glucose released by starch hydrolysis in the small intestine. This is a substantial difference and is probably the reason for the lower rates of lipogenesis (Goda et al. 1994, Mazur et al. 1990) and adipose tissue weights (Goda et al. 1994) in rats fed high amylose starches. It might have been expected that body weights would be lower in pigs fed the high amylose starches. In fact, no difference was detected and it may be beyond the power of the experiment to do so. Assuming that all starch escaped into the colon of pigs fed the high amylose diet, then the energy value of the diet would have been only 45% lower than that of the cornstarch diet. On this basis, the pigs would have gained 3.6 kg as opposed to the 8-kg gain of those fed the cornstarch diet. However, it has been reported by Muir et al. (1994) that only 30% of high amylose starch is measurable as "dietary fiber." On that basis, the high amylose diet would have provided 80% of the energy of the control diet. This would have led to a difference of 1 kg in weight gain—a difference too small to be measured with accuracy.

ACKNOWLEDGMENT

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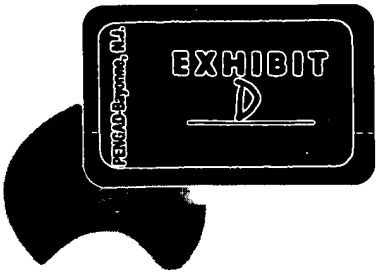

FOOD SCIENCE AUSTRALIA

A joint venture of CSIRO & the Victorian Government

CONFIDENTIAL PROJECT REPORT

PROBIOTIC GENETIC FINGERPRINTING

Project Number 111220

Report for:
Probiomics Ltd
Mr Kim Slatyer

Prepared by:
P. Scott Chandry

22 January 2007

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EXECUTIVE SUMMARY

The four *Lactobacillus fermentum* strains provided by Probiomics Ltd. (VRI 003 – NMI, VRI 003 – Medipharm, VRI 003/FII 511400, and KLD – Arla Foods) were submitted for restriction digest pattern analysis in order to determine the degree of relatedness between the strains. Genomic DNA was prepared for all of the strains and this was digested with four different restriction enzymes *Asc*I, *Pme*I, *Sgr*AI, and *Sma*I. Restriction digests were separated by pulsed-field gel electrophoresis then the relationships between strains were assessed by analysis of the resultant digestion patterns. Based on the results from restriction enzymes *Pme*I, *Sgr*AI, and *Sma*I no discernable differences were detected between the strains. Three different restriction patterns were detected following digestion with enzyme *Asc*I. The restriction patterns for VRI 003 – NMI and VRI 003 – Medipharm were identical while VRI 003/FII 511400 and KLD – Arla Foods varied from one another as well as the VRI 003 (NMI and Medipharm) pair.

CONFIDENTIAL PROJECT REPORT
PROBIOTIC GENETIC FINGERPRINTING

FOR Probiomics Ltd
Mr Kim Slatyer

1.0 RESEARCH TEAM'S QUALIFICATIONS

P. Scott Chandry and Sean C. Moore

Food Science Australia, Private Bag 16, Werribee VIC 3030, AUSTRALIA.

Food Science Australia (FSA) is a joint venture between the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and the Victorian Government. FSA conducts independent research for the Australian and Victorian Government, for Research and Development Corporations and for Australian and international food companies.

Work performed by:

- P. Scott Chandry
  - Senior project leader with 22 years of molecular biology research experience
  - Published peer reviewed article on characterization of non-starter micro-organisms by restriction analysis and pulsed-field gel electrophoresis (Chandry et al., 1998)
  - Analyzed over 1000 pulsed-field gel electrophoresis patterns
- Sean C. Moore
  - Research scientist with 15 years of molecular biology research experience
  - Published peer reviewed article on characterization of non-starter micro-organisms by restriction analysis and pulsed-field gel electrophoresis (Chandry et al., 1998)
  - Has prepared over 1000 pulsed-field gel electrophoresis samples

2.0 INTRODUCTION

Restriction fragment length polymorphism analysis was requested for four *Lb. fermentum* strains received from Probiomics Ltd (Table 1). The *Lb. fermentum* genome sequence has not been determined, so precise computer-based predictions of restriction digestion results were not possible. Instead, the G+C content of the genome was estimated from previously published work (Kandler and Weiss 1986) and the genome sequence of *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842 (an organism of similar G+C content) was used as a surrogate for computer-based restriction enzyme selection. Based on the predicted frequency of restriction site occurrence, restriction enzymes *Asc*I, *Pme*I, *Sgr*AI, and *Sma*I were selected for restriction fragment length polymorphism analysis (Table 2).

Table 1. Strain data provided by Probiomics Ltd.

Number	Strain	Strain Name	Location	Strain ID. No.	Form Supplied
1	*Lactobacillus fermentum*	VRI 003	NMI (formerly AGAL), VIC	NM02/31074	Agar Plate
2	*Lactobacillus fermentum*	VRI 003	Medipharm	PCC lactose powder = 06-517	Powder Sachet
3	*Lactobacillus fermentum*	VRI 003 / FII 511400	UNSW Culture Collection	CRC 511400, VRI003, 29/7/2004	Ampoule
4	*Lactobacillus fermentum*	KLD	ARLA Foods	*Lb. fermentum* KLD	Agar Plate

Table 2. Predicted frequency of restriction site occurrence

Restiction Enzyme	Predicted Fragments*
*Asc*I	15
*Pme*I	19
*Sgr*AI	121
*Sma*I	750

*Restriction enzyme cleavage frequency predicted from the genome sequence of *Lb. delbrueckii* subsp. *bulgaricus* ATCC 11842

A search of the scientific literature did not discover any published rules for interpretation of strain comparison by restriction fragment length polymorphism relevant to patented or proprietary strains. Therefore, the guidelines described by Tenover et al., (1995) and adopted by the CDC PulseNet (weblink below) were loosely applied to analysis of the *Lb. fermentum* strains. These rules have been used for previous research projects and are generally used for epidemiological tracking of pathogen outbreaks but not specifically for comparisons with proprietary strains.

3.0 RESULTS

The four strains were provided in a variety of forms (Table 1) but all were streaked onto agar plates to confirm the homogeneity of colony morphology. Several colonies were then selected at random for each strain and used to inoculate broth cultures in order to prepare the genomic DNA. Restriction digestion was performed on genomic DNA embedded in agarose blocks and the resultant restriction fragments separated by pulsed-field gel electrophoresis.

Restriction digestion with *Pme*I

The restriction patterns for all strains were identical when genomic DNA was digested with restriction enzyme *Pme*I (Fig. 1). This enzyme digested most of the strains into a pattern consisting of approximately 30 bands. None of the strains can be distinguished from one another with this restriction enzyme

Restriction digestion with *Sma*I

Similar to the results observed with *Pme*I, identical restriction patterns were observed for all strains following digestion of genomic DNA with restriction enzyme *Sma*I (Fig. 2). Digestion of *Lb. fermentum* with *Sma*I is predicted to generate approximately 750 fragments so electrophoretic separation was biased toward those of high molecular weight. If the complete range of fragments was visualized it would have yielded regions too dense in fragments to discriminate individual bands.

Restriction digestion with *Sgr*AI

Similar to the results observed with *Pme*I and *Sma*I, identical restriction patterns were observed for all strains following digestion of genomic DNA with restriction enzyme *Sgr*AI (data not shown). The quality of this gel was poor but no differences between the strains could be detected.

Restriction digestion with *Asc*I

The only restriction enzyme tested that was capable of discerning genetic differences between the strains was *Asc*I. This enzyme permitted the strains to be clustered into three different groupings labelled groups A – C (Figure 3, 4, and Table 3). Due to the complexity of the nomenclature for these strains they will be referenced using the group and number designations listed in Tables 1 and 3. The two strains in group A (#1, #2) are identical when digested with *Asc*I. The other two strains in groups B and C (#3 and #4 respectively) have slight differences from one another as well as the group A strains with fewer than four genetic changes separating all the groups (Table 4).

Table 3. Probiomics Ltd. strains organized by groupings of identical restriction patterns

Group	Number	Strain Name	Location
A	1	VRI 003	NMI (formerly AGAL), VIC
	2	VRI 003	Medipharm
B	3	VRI 003 / FII 511400	UNSW Culture Collection
C	4	KLD	ARLA Foods

Table 4. Number of differences observed between strain groupings*

	A	B	C
A	0	2	1
B	2	0	3
C	1	3	0

*Includes both bands lost and bands gained (e.g., a band that shifts downward results in a two changes when comparing between groups) (Tenover et al., 1995)

4.0 METHODS

Cell growth

Dried *Lb. fermentum* cells (~200 mg) supplied in capsules, and powder sachets were inoculated into 5.0 mL MRS broth (Oxoid), incubated 30 °C 18 hours. The contents of ampoules were resuspended in 300 µL MRS broth prior to addition to addition to a further 5.0 mL MRS broth and incubated 30 °C 18 hours. These cultures and those supplied on agar plates were streaked onto MRS agar plates then incubated at 30 °C for 18 hours in an anaerobic jar. Single colonies (3-6) were inoculated into 8.0 mL fresh MRS broth then incubated at 30 °C for 18 hours.

Genomic DNA preparation

4.0 mL of 18 hr culture cells were centrifuged at 13,100 rpm, 4 °C for 2 min. The supernatant was discarded and remaining cell pellets resuspended in 200 µL of 12 mM Tris pH 7.6, 10mg/mL lysozyme, 10mg/mL Lysostaphin. Removed a 100 µL aliquot and added 1.0 mL of 1.5% SeaPlaque low melting point agarose in TE buffer pH 7.6 (10mM Tris pH 7.6, 1mM EDTA) then placed into block molds. Slices from blocks were then transferred into EC lysis Buffer (6 mM Tris pH 7.6, 1 M NaCl, 0.1 M EDTA pH 7.5, 1% Sarkosyl) and incubated 30 min at 37 °C. Then transferred slices into ESP buffer (0.5 M EDTA pH 9.2, 1% Sarkosyl, 1 mg/mL Proteinase K) and incubated for 30 min at 50 °C. Slices were then washed with TE buffer containing 1 mM PMSF for 15 min at room temp. Genomic DNA slices were then washed a further two times in TE buffer without PMSF prior to rinsing with manufacturer specified restriction enzyme buffer. Restriction enzyme digestion was performed at the manufacturer recommended temperature overnight prior to electrophoresis.

Pulsed-field gel electrophoresis

Pulsed-field gel electrophoresis was performed with a CHEF Mapper (BioRad) using auto-algorithm settings for specified fragment lengths (see below). Following electrophoresis, gels were stained with ethidium bromide and imaged with a UV transilluminator. Digests were run with Roche PFGE Marker I Lambda ladder as a fragment length marker. Normalization and cluster analysis was performed on gel images with GelComparII.

Table 5. Restriction digestion conditions

Enzyme	*AscI*	*PmeI*	*SgrAI*	*SmaI*
Buffer	20 mM Tris Acetate pH 7.9 10 mM Magnesium Acetate 50 mM Potassium Acetate 1.0 mM Dithiothreitol	20 mM Tris Acetate pH 7.9 10 mM Magnesium Acetate 50 mM Potassium Acetate 1.0 mM Dithiothreitol 100 µg/mL BSA	20 mM Tris Acetate pH 7.9 10 mM Magnesium Acetate 50 mM Potassium Acetate 1.0 mM Dithiothreitol	33 mM Tris Acetate pH 7.9 10 mM Magnesium Acetate 66 mM Potassium Acetate 0.5 mM Dithiothreitol
Incubation conditions	37 °C 18 hr	25 °C 18 hr	37 °C 18 hr	25 °C 18 hr
Manufacturer	New England Biolabs	New England Biolabs	New England Biolabs	Roche

Table 6. Pulsed-field gel electrophoresis conditions

AscI	*PmeI*	*SgrAI*	*SmaI*
30-300kb	35-275kb	10-80kb	20-175kb
1% Agarose, 0.5 x TBE, 14 °C	1% Agarose, 0.5 x TBE, 14 °C	1% Agarose, 0.5 x TBE, 14 °C	1% Agarose, 0.5 x TBE, 14 °C
6V/cm	6V/cm	6V/cm	6V/cm
27:01 hr (NB: stopped ½ hour early)	27:07 hr	20:18 hr	26:56 hr
120 °	120 °	120 °	120 °
Switch times 2.16 – 26.29 sec	Switch times 2.53 – 24.04 sec	Switch times 0.47 - 6.80 sec	Switch times 2.98 - 15.11 sec
linear ramping	linear ramping	linear ramping	linear ramping

5.0 REFERENCES

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6.0 FIGURES

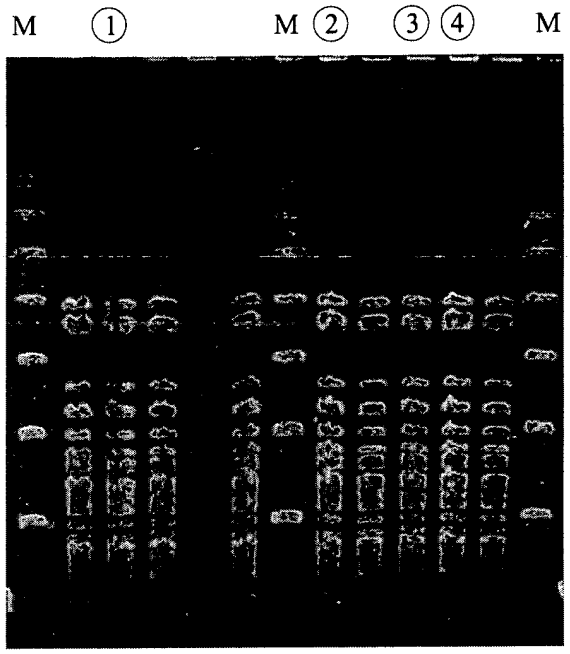

Figure 1. Image of *Pme*I restriction pattern without normalization. Lanes marked "M" contain marker DNA and lane numbering matches that used in Tables 1 and 3. Additional restriction patterns not relevant to this report are unlabeled.

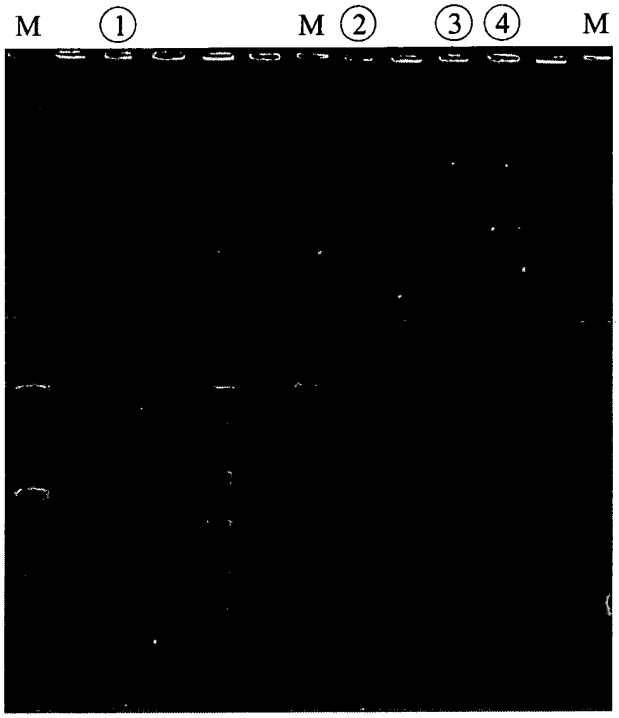

Figure 2. Image of *Sma*I restriction pattern without normalization. Lanes marked "M" contain marker DNA and lane numbering matches that used in Tables 1 and 3. Additional restriction patterns not relevant to this report are unlabeled.

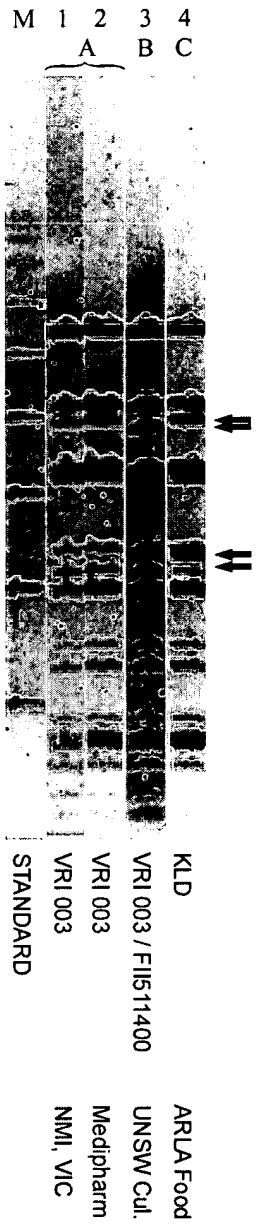

Figure 3. Normalized alignment of *Asc*I restriction patterns for Probiomics strains. Restriction pattern strips are listed with the strain name and location data provided by Probiomics. Lanes have been normalized then arranged to cluster most similar strains. Lanes marked "M" contain concatenated lambda phage marker DNA and letters and numbers above lanes matches those used in Tables 1 and 3. Text below the image denotes the strain name and location information presented in Table 1. Arrows indicate difference in the restriction patterns. The bracket above the restriction pattern strips designates a group of identical strains.

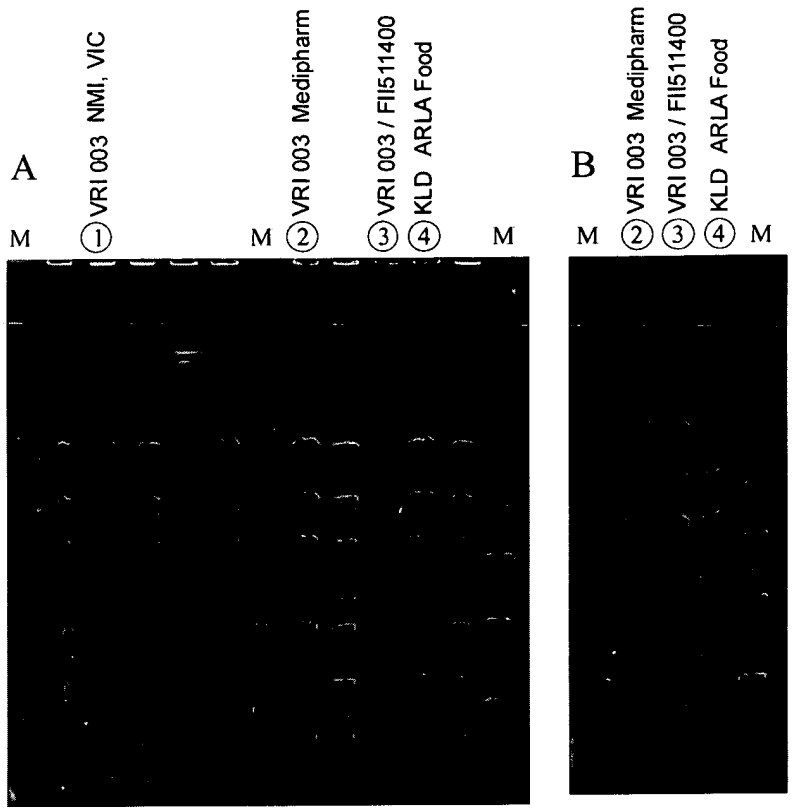

Figure 4. Images of *Asc*I restriction patterns without normalization. Lanes marked "M" contain marker DNA and lane numbering matches that used in Tables 1 and 3. Panel A is the image from the first pulsed-field gel while panel B is a repeat digest of selected strains to get an improved restriction pattern for lane 3. Additional restriction patterns not relevant to this report are unlabeled.

FOOD SCIENCE AUSTRALIA

A joint venture of CSIRO & the Victorian Government

DRAFT CONFIDENTIAL PROJECT REPORT

Antibiotic Resistance Profiles of *Lactobacillus fermentum* Strains

Project Number 111220

Report for:
Probiomics Ltd
Mr Kim Slatyer

Prepared by:
Ross Crittenden and Catherine McAuley

12 January 2007

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EXECUTIVE SUMMARY

The ability to transmit antibiotic resistance is recognised by Probiomics as an emerging safety consideration for its probiotic strains. Hence, this project was commissioned to compare the antibiotic susceptibility pattern of VRI-003 to that of other strains of *Lactobacillus fermentum* in order to determine if it possessed unusual antibiotic resistances that may indicate the presence of transferable antibiotic resistance genes. In this study, the minimal inhibitory concentration (MIC) of 12 antibiotics was investigated for 60 different *L. fermentum* isolates.

Antibiotic resistance was generally not observed for the *L. fermentum* strains when tested against ampicillin, chloramphenicol, clindamycin, erythromycin, linezolid, tetracycline and virginiamycin. The antibiotic resistance profiles of the VRI-003 isolates did show MIC values above the breakpoint values recommended for some antibiotics by the European Food Safety Authority (EFSA). However, the antibiotic resistances observed in the VRI-003 isolates were not unusual for *L. fermentum*. Resistance to kanamycin and streptomycin appears to be intrinsic rather than acquired, which is acceptable. Resistances to gentamicin, neomycin and trimethoprim require further examination. It is recommended, that with permission from Probiomics, the data be sent to an expert involved in the preparation of the EFSA guidelines on microbial antibiotic resistance to obtain his recommendations for the necessary steps to ensure that the antibiotic resistances observed in the current study are acceptable for a food grade organism.

About the Authors

Food Science Australia (FSA) is a joint venture between the Commonwealth Scientific and Industrial Research Organisation (CSIRO) and the Victorian Government. FSA conducts independent research for the Australian and Victorian Government, for Research and Development Corporations and for Australian and international food companies. Dr. Ross Crittenden is a Project Leader at Food Science Australia with 13 years of research experience in the field of probiotics. He holds a BScApp (hons) and a Ph.D from the University of Queensland in the field of Biotechnology. He is the author of more than 30 peer-review articles, 5 book chapters and 2 patents. Ms. Catherine McAuley is a food microbiologist who holds a B.Agr.Sc from the University of Melbourne. She has 10 years of research experience in microbiology including extensive expertise with antibiotic resistance testing.

CONFIDENTIAL PROJECT REPORT

Antibiotic Resistance Profiles of *Lactobacillus fermentum* Strains

1.0 INTRODUCTION

Probiomics Ltd is an Australian SME that is developing proprietary probiotic and biomolecular technology for commercial applications in consumer health, functional foods and pharmaceutical products. The main probiotic strain produced by Probiomics is *Lactobacillus fermentum* VRI-003, which now has accumulated health data from human clinical studies.

The ability to transmit antibiotic resistance is recognised by Probiomics as an emerging safety consideration for probiotic strains. Probiomics wishes to be proactive in supplying regulatory bodies with information concerning the antibiotic resistance profiles of its probiotic strains. Hence, this project was commissioned to compare the antibiotic susceptibility pattern of VRI-003 to that of other strains of *Lactobacillus fermentum* in order to determine if it possessed unusual antibiotic resistances that may indicate the presence of transferable antibiotic resistance genes. In this study, the minimal inhibitory concentration (MIC) of 12 antibiotics was investigated for 60 different *L. fermentum* isolates obtained from commercial samples provided by Probiomics and from internationally-recognised bacterial culture collections.

2.0 MATERIALS AND METHODS

The antibiotic testing regime applied in the current study was based on the recommendations of the European Food and Safety Authority (EFSA) for the assessment of bacteria for resistance to antibiotics of human or veterinary importance [1]. The twelve antibiotics used in this investigation (Table 1) are those (or analogues of those in the case of virginiamycin) recommended by EFSA. The antibiotic breakpoint values for MICs (growth above which is classified as resistance) listed in Table 1 are reproduced from the EFSA recommendations for lactobacilli [1].

The method used to determine the MIC of each antibiotic was based on the Clinical and Laboratory Standards Institute (CLSI www.clsi.org) method NCCLS M11-A6 [2], which is recognized as an approved method for antibiotic resistance testing by EFSA and the US FDA. Some modifications to this method were required for the growth of *L. fermentum*. These were the use of MRS agar rather than blood agar to grow the cultures; incubation of the plates for 24 h rather than 48 h, and use *Enterococcus faecalis* ATCC 29212 as a control strain since this strain grows on MRS and is used as the control in the CLSI antibiotic resistance test for aerobic organisms [3].

Sixty isolates of *L. fermentum* were obtained from Probiomics and from reputable culture collections. The isolates used and their sources are displayed in Table 2.

Table 1. Antibiotics used in the current study for antimicrobial resistance profiling of the *Lactobacillus fermentum* strains.

Antibiotic	**Abbreviation**	**Breakpoint concentration (µg/mL)***
Ampicillin	Amp	4
Chloramphenicol	Chlor	4
Clindamycin	Clin	4
Erythromycin	Ery	4
Gentamicin	Gen	8
Kanamycin	Kan	16
Linezolid	Lin	4
Neomycin	Neo	16
Streptomycin	Strep	16
Tetracycline	Tet	8
Trimethoprim	Trim	8
Virginiamycin – analogue for quinupristin + dalfopristin, sourced from Stafac 500 feed additive	Virg	4

* Recommended by the European Food and Safety Authority (EFSA) for resistance to antibiotics of human or veterinary importance [1].

3.0 RESULTS

The MIC values of the antibiotics for the *L. fermentum* isolates are displayed in Table 2. Cells that are shaded grey denote MIC values that are above the breakpoint values recommended by EFSA [1]. In general, antibiotic resistance was not observed for the *L. fermentum* strains when tested against ampicillin, chloramphenicol, clindamycin, erythromycin, linezolid, tetracycline and virginiamycin. The exceptions were strains CCUG 20884 and CCUG 32654 which showed strong resistance to ampicillin, indicating the presence of an antibiotic resistance mechanism, and mild resistance to tetracycline by CCUG 32662 and JCM 2768. The MIC values of tetracycline for CCUG 32662 and JCM 2768 were only one dilution above the EFSA breakpoint and are probably not indicative of an antibiotic resistance mechanism. The isolates equivalent to VRI-003 were not resistant to these antibiotics.

All isolates except one (NCIMB 700479) recorded MIC values for kanamycin that indicated resistance to this antibiotic. Similarly, only 2 *L. fermentum* isolates (CSCC 5403 and NCIMB 8962) registered sensitivity to streptomycin, in both cases at the EFSA breakpoint rather than below it. The almost universal resistance of *L. fermentum* isolates to these antibiotics suggest that this species is intrinsically resistant to kanamycin and streptomycin under the test conditions. Resistance to gentamicin, neomycin, streptomycin and trimethoprim was more variable between isolates. For each of these antibiotics the majority of isolates showed MIC values above the EFSA breakpoint MIC's, indicating that resistance to these antibiotics is a common trait in *L. fermentum*.

4.0 DISCUSSION

EFSA have proposed a decision tree to determine if an organism should be used as a feed or food ingredient (or additive) based on the nature of its antibiotic resistances [1]. This decision tree is shown in Figure 1. If the MIC values are less than or equal to the breakpoint values proposed by EFSA [1], then the organism is acceptable. This is clearly the case for VRI-003 isolates for ampicillin, chloramphenicol, clindamycin, erythromycin, linezolid, tetracycline and virginiamycin.

The almost universal resistance of the *L. fermentum* isolates to kanamycin and streptomycin indicate that resistance to these antibiotics is intrinsic rather than acquired, which is also acceptable.

The more variable resistance patterns seen to gentamicin, neomycin and trimethoprim require further examination. One possible explanation is interference by the test growth medium. The EFSA document [1] qualifies the breakpoint values it recommends for gentamycin, kanamycin, streptomycin, neomycin and trimethoprim with the statement that there is possible interference from the growth medium. Indeed, these are the same antibiotics that are notable for resistance by *L. fermentum* in current study. VRI-003 showed resistance to these antibiotics. However, this was not an unusual trait among *L. fermentum* strains. It is recommended that the data presented in this report be sent for evaluation by Prof. Atte von Wright, University of Kuipio, Finland, who provided advice to EFSA on the preparation of their recommendations document [1]. The questions posed would be:

1. Is it possible to test the antibiotic resistance patterns of the organisms using a different growth medium and still use the EFSA breakpoints given that they were determined using MRS agar?
2. Is there a need to characterise the genetic basis of the resistance, or is the fact that the majority of strains showed resistance indicate intrinsic resistance?
3. Are further steps required to confirm the safety of VRI-003 in terms of transferable antibiotics resistances?

One final factor which may account for some variability is the reliability of the taxonomy of the strains used in the current study. These have mostly been obtained from reputable culture collections. However, the basis on which the taxonomy has been assigned for each strain should be explored if possible.

5.0 CONCLUSIONS

- The antibiotic resistance profiles of the VRI-003 isolates show MIC values above the breakpoint values recommended for some antibiotics by EFSA [1].
- However, the antibiotic resistances observed in the VRI-003 isolates were not unusual for *L. fermentum*.
- Resistance to kanamycin and streptomycin appears to be intrinsic rather than acquired, which is acceptable.
- Resistances to gentamicin, neomycin and trimethoprim require further examination and problems with the standard methodologies when applied to this organism can not be ruled out.

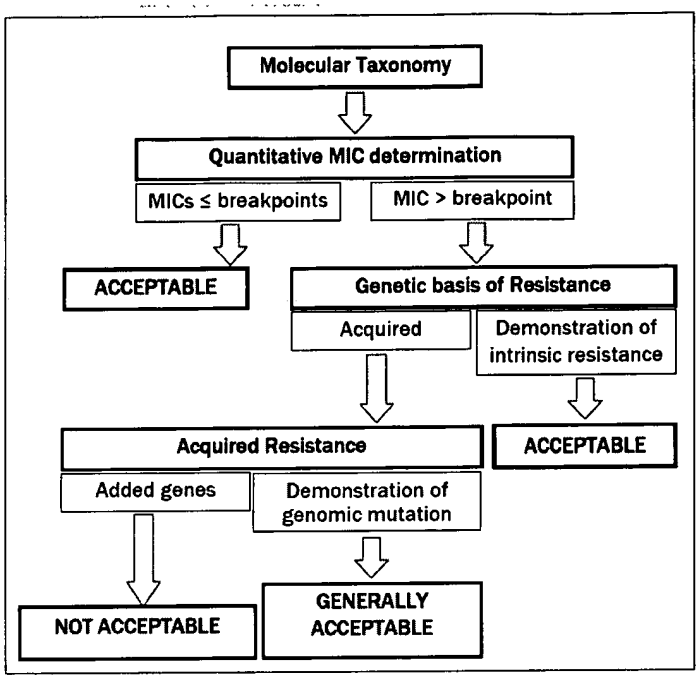

Figure 1. Proposed scheme for the antimicrobial resistance assessment of a bacterial strain used as a food or feed additive.

6.0 RECOMMENDATION

It is recommended that the data presented in this report be sent for evaluation by Prof. Atte von Wright, University of Kuipio, Finland, who provided advice to EFSA on the preparation of their recommendations document [1]. The questions posed would be:

1. Is it possible to test the antibiotic resistance patterns of the organisms using a different growth medium and still use the EFSA breakpoints given that they were determined using MRS agar?
2. Is there a need to characterise the genetic basis of the resistance, or is the fact that the majority of strains showed resistance indicate intrinsic resistance?
3. Are further steps required to confirm the safety of VRI-003 in terms of transferable antibiotics resistances?

Table 2. Minimum Inhibitory Concentrations (MIC) of antibiotics of food or veterinary importance for *Lactobacillus fermentum* isolates.

			MIC (μg/mL)											
	Strain	Strain ID No.	Amp	Chlor	Clin	Ery	Gen	Kan	Lin	Neo	Strep	Tet	Trim	Virg
1	*Lb fermentum*	[a]NM02/32959	≤0.125	4	≤0.125	0.5	32	≥128	2	64	≥128	4	16	0.5
2	*Lb fermentum*	[a]NM02/31074	≤0.125	4	≤0.125	0.5	32	≥128	2	32	≥128	4	16	0.5
3	*Lb fermentum*	[g]VRI 003 Probiomics	≤0.125	4	≤0.125	0.5	32	≥128	2	32	≥128	4	16	0.5
4	*Lb fermentum*	[a]NM02/32958	0.5	4	≤0.125	1	32	≥128	2	32	128	4	≥64	0.25
5	*Lb fermentum*	[b]NCIMB 40646/PAT134	≤0.125	4	≤0.125	0.5	32	≥128	2	64	≥128	4	16	0.5
6	*Lb fermentum*	[b]NCIMB 5221	≤0.125	2	≤0.125	0.25	32	≥128	1	32	128	1	2	≤0.125
7	*Lb fermentum*	[b]NCIMB 7230	≤0.125	2	≤0.125	≤0.125	8	128	1	16	32	1	4	≤0.125
8	*Lb fermentum*	[b]NCIMB 8828	0.25	4	≤0.125	0.5	16	≥128	2	32	128	2	16	0.5
9	*Lb fermentum*	[b]NCIMB 8961	≤0.125	2	≤0.125	0.25	4	128	1	16	32	2	2	≤0.25
10	*Lb fermentum*	[b]NCIMB 12116	≤0.125	4	≤0.125	0.5	8	128	2	16	64	4	16	≤0.25
11	*Lb fermentum*	[b]NCIMB 700335	≤0.125	4	≤0.125	0.5	32	≥128	2	32	128	4	32	0.5
12	*Lb fermentum*	[b]NCIMB 700928	0.25	4	≤0.125	≤0.125	16	≥128	2	32	128	4	8	0.25
13	*Lb fermentum*	[b]NCIMB 701052	0.25	4	≤0.125	0.5	16	≥128	2	32	128	4	64	0.5
14	*Lb fermentum*	[b]NCIMB 701751	≤0.125	4	≤0.125	0.5	16	≥128	2	16	128	2	4	0.25
15	*Lb fermentum*	[b]NCIMB 702342	≤0.125	4	≤0.125	0.5	16	≥128	2	16	128	4	64	0.5
16	*Lb fermentum*	[b]NCIMB 40643	0.5	4	≤0.125	1	32	≥128	2	32	128	8	≥64	≤0.25
17	*Lb fermentum*	[b]NCIMB 40644	≤0.125	4	≤0.125	0.25	16	≥128	1	32	64	4	≥64	≤0.25
18	*Lactobacillus spp*	[b]NCIMB 40647	≤0.125	4	≤0.125	0.5	32	≥128	2	64	≥128	4	16	0.5
19	*Lactobacillus spp*	[b]NCIMB 40648	0.25	4	2	4	≥64	≥128	1	≥128	≥128	0.25	≥64	1
20	*Lb fermentum*	[c]CSCC 5173	≤0.125	4	≤0.125	0.5	16	≥128	2	16	128	4	64	0.5
21	*Lb fermentum*	[c]CSCC 5174	0.5	2	≤0.125	0.25	8	128	1	16	32	2	2	≤0.25

	Strain	Strain ID No.	MIC (μg/mL) Amp	Chlor	Clin	Ery	Gen	Kan	Lin	Neo	Strep	Tet	Trim	Virg
22	*Lb fermentum*	[c]CSCC 5175	≤0.125	2	≤0.125	0.5	32	≥128	2	64	≥128	2	16	≤0.25
23	*Lb fermentum*	[c]CSCC 5216	≤0.125	4	≤0.125	0.5	32	≥128	2	32	≥128	4	8	≤0.25
24	*Lb fermentum*	[c]CSCC 5217	0.25	4	≤0.125	0.5	16	32	2	32	128	4	32	0.5
25	*Lb fermentum*	[c]CSCC 5362	≤0.125	4	≤0.125	0.5	32	≥128	2	64	≥128	4	16	0.5
26	*Lb fermentum*	[c]CSCC 5403	≤0.125	2	≤0.125	0.25	2	16	1	8	16	0.5	8	≤0.25
27	*Lb fermentum*	[b]NCIMB 702341	≤0.125	4	≤0.125	0.5	16	≥128	2	16	128	4	32	0.5
28	*Lb fermentum*	[b]NCIMB 8829	0.25	4	≤0.125	0.5	32	≥128	2	32	128	2	64	≤0.25
29	*Lb fermentum*	[b]NCIMB 8830	≤0.25	4	≤0.125	0.5	32	≥128	2	32	128	2	≥64	0.25
30	*Lb fermentum*	[b]NCIMB 8962	≤0.125	2	≤0.125	0.25	8	128	1	32	16	2	2	0.25
	E. faecalis	[d]ATCC 29212	0.25	4	64	8	64	≥128	1	≥128	≥128	8	≥64	8
	inoculum repeat	[d]ATCC 29212	0.25	4	64	8	≥64	≥128	1	≥128	≥128	8	≥64	8
31	*Lb fermentum*	[b]NCIMB 700479	0.25	4	≤0.125	0.25	2	8	2	8	32	2	4	0.25
32	*Lb fermentum*	[b]NCIMB 700927	0.25	4	≤0.125	0.5	16	≥128	2	32	128	4	4	0.5
33	*Lb fermentum*	[b]NCIMB 701068	≤0.125	4	≤0.125	0.5	32	≥128	2	32	≥128	2	64	0.25
34	*Lactobacillus spp. (Ogi)*	[b]NCIMB 12117	0.25	4	≤0.125	0.25	16	≥128	2	32	64	4	≥64	0.25
35	*Lb reuteri*	[b]NCIMB 11951	1	2	≤0.125	0.25	16	≥128	2	32	64	8	≥64	0.5
36	*Lb fermentum*	[e]CCUG 20884	≥64	4	≤0.125	0.25	16	≥128	2	32	128	2	16	0.25
37	*Lb fermentum*	[e]CCUG 32654	≥64	4	≤0.125	0.25	16	128	2	32	64	2	4	≤0.125
38	*Lb fermentum*	[e]CCUG 32662	1	2	≤0.125	0.25	16	≥128	2	32	128	16	64	0.5
39	*Lb fermentum*	[e]CCUG 38292A	1	4	≤0.125	1	16	≥128	2	32	128	4	32	0.5
40	*Lb fermentum*	[e]CCUG 47616A	≤0.125	2	≤0.125	0.25	≥64	≥128	1	≥128	≥128	2	4	0.25
41	*Lb fermentum*	[e]CCUG 48099	≤0.125	4	≤0.125	0.25	16	128	2	16	64	4	64	0.25

	Strain	Strain ID No.	MIC (μg/mL) Amp	Chlor	Clin	Ery	Gen	Kan	Lin	Neo	Strep	Tet	Trim	Virg
42	*Lb fermentum*	[e]CCUG 49999	0.25	4	≤0.125	0.5	32	≥128	2	64	≥128	4	8	0.25
43	*Lb fermentum*	[e]CCUG 52483	0.25	4	≤0.125	0.5	8	64	2	16	32	2	8	0.25
44	*Lb fermentum*	[f]JCM 2761	0.5	4	≤0.125	0.5	2	64	2	8	32	4	≥64	0.25
45	*Lb fermentum*	[f]JCM 2766	0.25	4	≤0.125	0.25	16	≥128	2	16	64	4	16	0.25
46	*Lb fermentum*	[f]JCM 2767	0.25	2	≤0.125	0.25	32	≥128	1	32	128	2	8	0.25
47	*Lb fermentum*	[f]JCM 2768	1	4	≤0.125	0.25	32	≥128	1	32	≥128	16	≥64	0.5
48	*Lb fermentum*	[f]JCM 5867	≤0.125	4	≤0.125	0.5	16	≥128	2	32	128	4	≥64	0.25
49	*Lb fermentum*	[f]JCM 5868	1	4	≤0.125	1	16	≥128	2	32	64	8	≥64	0.5
50	*Lb fermentum*	[f]JCM 5869	0.5	4	0.25	1	16	≥128	4	16	64	8	≥64	1
51	*Lb fermentum (reuteri)*	[f]JCM 1081	1	4	≤0.125	0.5	16	≥128	2	32	128	4	≥64	0.5
52	*Lb fermentum (reuteri)*	[f]JCM 1084	1	4	≤0.125	1	4	128	2	8	16	4	4	0.5
53	*Lb fermentum (reuteri)*	[f]JCM 1088	≤0.125	1	≤0.125	≤0.125	32	≥128	1	64	16	2	16	≤0.125
54	*Lb fermentum*	[g]CRC 511400, VRI003	≤0.125	4	≤0.125	0.5	32	≥128	2	64	≥128	4	16	0.5
55	*Lb fermentum*	[g]Arla KLD	≤0.125	4	≤0.125	0.5	32	≥128	2	32	≥128	4	16	0.5
56	*Lb fermentum*	[g]lactose powder 06-517	≤0.125	4	≤0.125	0.5	32	≥128	2	64	≥128	4	16	0.5
57	*Lb fermentum*	[g]trehalose powder 06-568	≤0.125	4	≤0.125	0.5	32	≥128	2	64	≥128	4	16	0.5
58	*Lb fermentum*	[g]Clinical Trial VRI 065/B	≤0.125	4	≤0.125	0.5	32	≥128	2	64	≥128	4	16	0.5
59	*Lb fermentum*	[g]ProBioPCC /VRI 036 E1	≤0.125	4	≤0.125	0.5	32	≥128	2	64	≥128	4	16	0.5
60	*Lb fermentum*	[g]ACTIVE capsules	≤0.125	4	≤0.125	0.5	32	≥128	2	64	≥128	4	16	0.5
	E. faecalis	[d]ATCC 29212	**0.25**	**4**	**64**	**8**	**≥64**	**≥128**	**1**	**≥128**	**≥128**	**16**	**≥64**	**8**

Legend for Table 2.

Cells that are shaded grey indicate MIC values that are above the breakpoint MIC values recommended for lactobacilli by the European Food Safety Authority [1].

a – National Measurement Institute, Melbourne VIC, Australia.

b – The National Collection of Industrial, Marine and Food Bacteria, Aberdeen, Scotland.

c – CSIRO Starter Culture Collection, held at the Australian Starter Culture Research Centre, Melbourne VIC, Australia.

d – American Type Culture Collection, Manassas VA, USA.

e – Culture Collection, University of Göteborg, Sweden

f – Japan Collection of Microorganisms, Saitama, Japan.

g – Commercial cultures provided by Probiomics Ltd.

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